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The high level expression in some breast cancers of HER-2/neu, correlates with poor prognosis. An antisense agent capable of down-regulating expression of this gene should be useful in treating these cancers. To achieve high avidity and specificity, we designed chimeric antisense molecules consisting of a short active DNA fused to a short "anchor" 2'-O-methyl RNA complementary to non-contiguous single-stranded sequences in the 5'-UTR of HER-2/*neu*. DNA hexamers were tested for their ability to mediate RNaseH cleavage. The two most potent sequences were chosen as the active and anchor partners, and they were linked using spacers of various lengths. Besides these dimeric antisense agents, we attempted to further increase potency with trimeric oligonucleotides comprising 1 "active" and 2 "anchor" oligonucleotides complementary to 3 non-contiguous sequences. We tested the ability of both dimeras and trimeras to down-regulate expression of the luciferase reporter gene placed downstream of the HER-2/neu 5'-UTR in wheat germ cell-free extracts. While dimeras successfully decreased expression at 1μM, trimeras, surprisingly, were stimulatory at 1μM for luciferase activity. Since both were equally potent in promoting RNA degradation of HER-2/neu mRNA in the RNaseH assay, this finding could be a clue to the mechanism that is responsible for HER-2/neu overexpression.We are now examining this phenomenon in breast cancer cell lines. In separate studies, we have developed and obtained a United States patent on a carrier polymer that can potentially provide oral bioavailability and enhanced cellular/cytosolic uptake into cells for antisense therapeutics.

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Introduction:

HER-2/neu, encoded by the erbB-2 gene, is a member of the epidermal growth factor receptor family whose high level of expression in some breast cancers and other malignancies correlates with poor prognosis (Hynes and Stern, 1994; Reese and Slamon, 1997). Inhibition of HER-2/neu expression by the therapeutic antibody Herceptin has proven to be clinically useful in treating breast cancer (Ross and Fletcher, 1999). An antisense agent capable of down-regulating expression of this gene should be useful in treating these cancers. Antisense DNA agents can theoretically act by various mechanisms, but appear to exert most of their effect by hybridization to their RNA targets, resulting in the destruction of these targets by cellular RNaseH.

As a new approach to the design of antisense agents with high affinity and specificity for the 5'-untranslated region (UTR) of HER-2/neu mRNA, we have designed bifunctional agents. These agents consist of a short "active" hexameric oligonucleotide binding to a sequence in the target UTR, coupled to a short "binding" 2'-O-methyl RNA hexamer with affinity for another portion of the UTR. The most active short DNA oligonucleotide tested was designated as the "active" hexamer and linked to the second, or in some cases, a third most active oligonucleotide, designated as the "binding" hexamer, through a carbon spacer of various lengths. These "chimeric" antisense molecules, both dimeric and trimeric, have shown to markedly stimulate RNaseH sensitization at the site targeted by the active oligonucleotide.

A major problem in using antisense oligonucleotides with phosphorodiester bonding in drug design is that these antisense compounds are nuclease sensitive, and thus, are rapidly degraded by both exonucleases and endonucleases in cells. (Agrawal et al, 1997)The first generation of modified antisense oligonucleotides was phosphorothioate oliogonucleotides. A distinct advantage with these molecules is their resistance to nucleases while still retaining the ability to promote RNaseH cleavage of the target RNA (Agrawal et al, 1997). However, studies have shown that phosphorothioate modified oligonucleotides have a lower affinity for its complementary sequence and result in diminished antisense activity (Agrawal et al, 1997). We constructed dimeric and trimeric antisense compounds with a phosphorothioate modified "active" hexamer and tested their ability to promote RNaseH mediated cleavage in vitro. We confirmed that phosphorothioate modified antisense oligonucleotides are less potent stimulators of RNaseH than unmodified DNA oligonucleotides.

We continued our novel approach towards antisense design by constructing a third hexamer and attaching the hexamer to our most potent dimeric compound. The third hexamer was constructed as a second "binding" 2'-O-methyl RNA hexamer with affinity for another portion of the 5'-untranslated region of the HER2 mRNA. The hexamer was attached to the dimeric compound through various 9-carbon spacers. The resulting trimeric antisense compound was also shown to markedly stimulate RNaseH sensitization at the site targeted by the active oligonucleotide, but not with greater affinity than that of the most potent dimeric compound.

Based on the RNaseH data, we evaluated our dimeric and trimeric compounds in coupled T7 polymerase wheat germ cell-free extracts. Wheat germ cell-free extracts produce very high levels of protein and is useful in evaluating the effectiveness of antisense molecules via RNaseH. We prepared a construct that contained the HER2 5'-UTR upstream of the luciferase reporter gene driven by the T7 promoter. Our dimeric and trimeric antisense compounds were tested for their ability to decrease expression by measuring luciferase activity. Our best dimeric compound, Dimera3-PS, inhibited expression of the T7/HER-2/luciferase construct but had no effect in the control T7/Luciferase plasmid, which lacks the HER-2 5'-UTR. Our trimeric antisense compounds however, exhibited stimulation in translation of the HER2/luciferase construct, while showing no effect in the control construct.

Body:

Creation of a peptide through combinatorial synthesis that binds to HER-2/neu mRNA

We have previously studied peptides that bind specifically to RNA regulatory elements, with binding depending on RNA structure (Wang et al., 1995; Choudhury et al., 1998,1999). To develop the binding element of the bifunctional agent, one approach has been to prepare small libraries conformationally constrained cyclic peptides were prepared on SPOTs membranes, to determine which peptide has optimized specificity of binding to the 5'-UTR of HER-2/neu mRNA. SPOTs membranes are cellulose based and provide a platform for the construction of custom peptide libraries. The cyclic design, shown in Figure 1, provides 6 positions that were varied in the synthesis by adding synthetic (not natural) amino acids whose abbreviations and structures are shown in Figure 2. The amino acids in all six variable positions in the starting peptide to be deconvoluted were D-Arg. The binding assay was performed in PBS by using 10 ng of 5'-[^{32}P]-HER-2 mRNA consisting of 165 bases of the 5'-UTR region in the presence of 10 mg of competitor yeast RNA.

The initial peptide library was deconvoluted at positions X1 and Y1. The SPOTs membrane was placed in a phosphor-imager to determine relative affinities of each peptide to HER-2/neu mRNA. The position on the membrane which has the most intense spot corresponds to the spot on the membrane with the greatest amount of radioactivity, and hence the greatest amount of HER-2/neu mRNA which has bound to a peptide. Binding of HER-2/neu mRNA to a given peptide on the membrane was expressed as percent of RNA bound. Results in Table 1 show that the peptide containing L-hLys in position Y1 and Ala-3-pyr in position X1 exhibited the second strongest affinity for the HER-2/neu mRNA among those tested.

A new peptide library was constructed by fixing positions X1 and Y1 and varying amino acids in the X2 and Y2 positions. The binding study was repeated using the same concentrations of both HER-2/neu mRNA and yeast RNA. Table 2 shows the results of the binding assay. The strongest binding peptide containing Ala-4-pip in the Y2 position and Gly-4-pip in the X2 position was used as the fixed amino acids in the third deconvoluted peptide library.

The third peptide library was constructed varying the position X3 and Y3. The binding was repeated using the third peptide library and the results of the binding study are shown in Table 3. The optimal peptide for binding specificity by this assay is shown in Figure 3. Deconvolution cycles could be contained at each position using the same or different amino acids. Further studies are planned to attach a HER-2/neu antisense DNA to the best peptide and test the effect of this peptide on binding to the target RNA and on inhibition of HER-2/neu expression in a model system.

HER-2/neu Sequence Analysis:

Our goal is to target the 5' untranslated region of the HER-2 mRNA, and to perform in vitro studies with antisense oligonucleotides to develop agents capable of decreasing in expression of HER-2 in cancer cells. In order to generate RNA for in vitro studies, we amplified the 5' end of the HER-2 gene. Although multiple studies have yielded the same sequence for the ORF region of the HER-2 gene five studies have reported four different sequences for the 5' UTR and promoter region of the HER-2 gene. (Child et al., 1999; Hudson et al., 1990; Coussens et al., 1985; Tal et al., 1987; Yamamoto et al., 1986)

The HER-2 sequence was first reported by Coussens et al. (1985; Genebank accession number M11730). The sequenced DNA was derived from an isolated λgt10 human genomic cDNA clone detected by southern blot hybridization with oligonucleotide probes for the transforming gene of the avian erythroblastosis virus, v-erbB. This sequence does not contain the entire 5' untranslated region of the HER-2 gene, but only 150

bases of the 178 bases that constitute the entire 5' untranslated region, and diverges from the sequence reprted by Child et al. (1999) at base –96 (with the A of the translation initiating AUG, set at +1).

The sequence with GeneBank accession number X03363 (Yamamoto et al., 1986) was derived from an isolated breast cancer cell line cDNA library and detected by probing with a DNA fragment prepared from a c-erbB-2 genomic clone. This sequence contains 174 of the 178 bases that constitute the entire 5' untranslated region of the HER-2 gene. This sequence diverges from the sequences of Child et al. (1999) at position 141 of the UTR.

Later erbB-2 work focused on the overexpression of the HER-2 gene in breast cancer cells. Tal et al. (1987) verified the location of the HER-2 promoter region by sequence analysis of genomic and cDNA clones, and by nuclease S1 protection analysis. The promoter region was isolated by screening a λn2 human genomic library, with fragments from HER-2 cDNA with the sequence of the isolated clone was reported to Genebank under the accession number M16789. This sequence is 757 bases long and contains both the 5' untranslated region and the upstream promoter region of the HER-2 gene. This sequence matches the sequence of Child et al. (1999) I the 5' untranslated region, but diverges in the promoter region.

Hudson et al. (1990; GeneBank accession number J05264) reported a sequence derived from a genomic library from human white blood cells, screened with synthetic oligonucleotides representing three known sequences in the upstream region of the HER-2 gene plus the promoter regions upstream of the transcription start site. The sequence J05264 matches the M16789 sequence and our sequence for the entire 5' untranslated region but diverges 51 bases upstream from the transcription start site.

We determined the sequence of PCR amplified human female genomic DNA (Promega), using PCR primers described by Child et al. (1999). The upstream primer was 5'-TTAAGGATCCGTGGAGGAGGAGGGCTGCTT-3' (labeled as solid arrows, Figure 4), and the downstream primer, 5'-GGGCAAGAGGGCGAGGAG-3', corresponding to the open reading frame of exon 1. The resulting PCR product was then sequenced via an automated DNA sequencer. The results are shown in Figure 4. The same ORF primer failed to amplify DNA from the same template preparation when used in combination with the upstream primers indicated in dashed arrows that are reported from other divergent 5'-UTR sequences.

The amplified PCR product of the 5'untranslated region was also achieved using primers derived from sequence J05264. We have confirmed the 5' UTR sequence of HER-2 stated in Child et al. (1999) in several breast cancer cell lines. HER-2 overexpressers, SK-BR-3, BT549, T47D, ZR-75-1, and BT474 as well as low HER-2 expressers BT-20 and MCF-7, were cultured and their DNA extracted via Proteinase K treatment. Genomic DNA from each cell line was analyzed via PCR amplification. We used upstream primers specific for each reported sequence in the literature while maintaining the same downstream primer complementary to exon 1 to determine which 5' untranslated region existed in these breast cancer cell lines. All of the cell lines tested yielded a PCR product of predicted length using PCR primers from Child et al. (1999) Therefore, we conclude that our cell lines, indeed, contain that sequence in the 5'-UTR of HER-2. We have no explanation for the divergent sequences reported elsewhere.

HER-2/neu mRNA structure:

In order to identify possible loops in the nascent HER-2/neu RNA, the 5' untranslated region of the HER-2/neu mRNA sequence was subjected to the Wisconsin GCG mfold software program. This program determines possible secondary structures of RNA based on predicted energy levels. Figure 5 shows one such 2-dimensional folding of the HER-2/neu mRNA. Single stranded regions are predicted to be the best binding sites for antisense DNA (Ho et al., 1996; Wrzesinski et al., 2000). In order to determine whether these loops do indeed exist, oligonucleotides were constructed that were complementary to the putative loops provided by the mfold program. DNA hexamers were chosen to target the putative loops in the mRNA structure, labeled in yellow. The 5'-[32P]-end-labeled HER-2/neu mRNA consisting of the first 165 bases of the UTR was

subjected to RNaseH digestion in the presence of a set hexamer antisense oligonucleotides complementary to specific regions of the UTR.

The results of this RNaseH sensitization assay are shown in Figure 6. DNA hexamers complementary to loop1 and 5 failed to sensitize their targets to RNaseH, suggesting that these putative loops did not exist or were not accessible in that region of the HER-2/neu RNA. Hexamers (1uM and 10uM) complementary to loops 2, 3, 4 and 6 did sensitize the RNA to RNaseH, confirming that these loops do exist in the 5' untranslated region of the HER-2/neu mRNA. The relative strength of each hexamer to induce cleavage by RNaseH was determined by decreasing the concentrations of the hexamer used in the RNaseH assay. The results in Figure 7 show that the hexamer complementary to loop 3 resulted in the most cleavage of the HER-2/neu mRNA at 0.1μM and 1μM as indicated by production of the cleaved RNA fragment and disappearance of the precursor RNA.

Proving the utility of using DNA hexamers to confirm the existence of the putative loops of the 5'-UTR of the HER-2/neu RNA, we used DNA hexamers to confirm the rest of the putative loop regions as predicted by the RNA folding program. The results in Figure 8 show additional locations in the HER-2 mRNA 5'-UTR where single stranded regions exist. Hexamers (10μM) complementary to loops 7, 8, and 9 did sensitize the RNA to RNaseH, confirming that these loops do exist in the 5' untranslated region of the HER-2 mRNA. While the RNA folding program can determine possible single stranded regions in RNA and by extension, accessible sites for antisense binding, it is unlikely to identify all single stranded regions.

Our attempt is to identify the most accessible regions in the 5' untranslated region of the HER-2 mRNA to cleavage by RNaseH in response to antisense binding. In order to determine whether we have identified all of the most accessible sites, we conducted an RNaseH sensitization assay using a random hexamer oligonucleotide library (produced by IDT, Inc.). Figure 9 shows the random oligonucleotide library yielded cleavage products that correspond closely to the most sensitive sites previously determined by the RNA folding program.

Enhanced Sensitivity of HER-2/neu mRNA to RNase H by Chimeric Oligonucleotides

As an alternative approach to developing low molecular weight antisense agents specific for the 5'UTR of HER-2/neu mRNA, we synthesized bifunctional agents coupling an active site DNA antisense hexamer with a 2'-O-methyl RNA structure was selected because such antisense molecules bind to their target even more tightly than do DNA molecules, but they fail to sensitize the target to RNaseH and they themselves are not sensitive to DNases or RNases. It has previously been shown that two tethered oligonucleotides binding to nearby targets on an RNA molecule can act synergistically to enhance binding affinity provided the two binding elements are linked in the appropriate geometric arrangement (Moses et al., 1997). Therefore, we tested a series of chimeric antisense molecules against the 5'-UTR of HER-2 mRNA. These chimeras, to be referred to as dimeras, consisted of a DNA hexamer (active site) targeted to loop 2, and a 2'-O-methyl RNA hexamer (binding site) targeted to loop 3, linked by carbon spacers of various lengths (0, 1, 2, 3, or 4 9-carbon units).

In our first study in this series, two dimeric molecules were used in an in vitro RNaseH assay to determine the sensitivity of the HER-2 mRNA molecule to RNaseH relative to that of the active site hexamers alone. The dimeric molecules consist of two hexameric oligonucleotides chemically linked to each other and oriented in a 5' to 3' direction. The 5' end of the dimeric molecule consists of a 2'-O-methyl RNA hexamer complementary to loop 3 in the HER-2 mRNA and the 3' end of the molecule is a DNA hexamer which is complementary to loop2. Varying lengths of spacers consisting of repeating units of nine carbons in length attach the two hexamers, linking together the 3' end of the 2'-O-methyl RNA to the 5' end of the DNA hexamer. A schematic representation of our antisense design is shown in Figure 10. In this experiment, two

dimeric molecules were constructed differing in the number of spacers adjoining the two oligonucleotides, one 9-carbon spacer (Dimera 1) and three 9-carbon spacers (Dimera 3), respectively.

The 5'[32P]-labeled HER-2 mRNA was subjected to RNaseH digestion in the presence of the dimeric molecules, and the extent of cleavage of HER-2 RNA was compared to reactions containing equal concentrations of the DNA hexamer complementary to loop 2. The results in Figure 11 show that dimeric molecules with both linkers dramatically increased the sensitivity of HER-2 mRNA to RNaseH at all concentrations tested compared to the DNA hexamer alone. Significant cleavage was observed for both dimeric molecules at 0.01μM and 0.001μM, with the dimeric molecule containing three 9-carbon spacers exhibiting greater sensitization to RNaseH then did the dimera with only one spacer. The DNA hexamer alone at 0.01μM and 0.001μM failed to promote cleavage of the RNA molecule. The dimeric molecule containing one 9-carbon spacer resulted in a slight increase at another site distinct from the target site. This is not true for the dimeric molecule with three spacers. It appears from these results that dimeric molecules show increased ability to sensitize the HER-2 mRNA to RNaseH mediated cleavage, with cleavage occurring preferentially for the targeted loop.

Sensitiziation of HER-2/neu mRNA to RNaseH cleavage is dependent on the length of the carbon spacers on the dimeric molecule

In order to further determine which linker length would result in the greatest sensitization of HER-2/neu mRNA to RNaseH activity, dimeric antisense molecules containing 0-4 9-carbon spacers linking the two components were compared. The results in figure 12 show that the dimeric molecules of all lengths sensitized HER-2/neu mRNA to RNaseH cleavage at all of the concentrations tested. The degree of HER-2/neu mRNA sensitization to RNaseH activity was dependent on the size of the spacer between the two antisense oligonucleotides. Dimeric molecules either lacking a spacer or containg three 9-carbon spacers resulted in the greatest sensitization of HER-2/neu mRNA to RNaseH activity at a concentration of 1nM, as indicated by production of the cleaved RNA fragment and by disapprearance of the precursor RNA. Dimeric antisense molecules of all spacer sizes tested enhanced HER-2/neu mRNA senisitization to RNaseH when compared to the DNA hexamer alone.

Phosphorothioate modified antisense compounds are less effective in promoting RNaseH mediated cleavage of the 5' untranslated region of HER-2 mRNA

DNA based antisense oligonucleotides have been shown to be sensitive to both endonucleases and exonucleases in cells. (Agrawal et al., 1997) Antisense modifications, including phosphorothioates, show increased resistance to nucleases while maintaining their ability to bind to a complementary RNA target and promote RNaseH mediated cleavage of the RNA molecule. (Agrawal et al., 1997) We modified our "active" portion of the best dimeric compounds, the DNA hexamer, to produce a phosphorothioate oligonucleotide. The resulting compounds are labeled Dimera0-PS and Dimera3-PS. The rest of the compound remained unchanged. The 5'-[32P]-labeled HER-2 5'-UTR of mRNA was subjected to RNaseH digestion in the presence of DNA and phosphorothioate (PS) dimeric antisense molecules, and the extent of cleavage of HER-2 RNA was determined by examining degradation of the of the labeled mRNA on a denaturing polyacrylamide gel. Our results, shown in Figure 13, confirm that, indeed, phosphorothioate modified antisense compounds are less potent stimulators of RNaseH at both 0.1and 0.01μM when compared to DNA. Antisense compounds Dimera0 and Dimera3, with zero 9-carbon spacers and three 9-carbon spacers respectively, were approximately equivalent in their ability to promote RNaseH mediated cleavage of the HER2 mRNA molecule. Figure 14 also shows the comparison between Dimera0 and Dimera3 in the presence of competing total yeast RNA at 1μg/ul and 5μg/ul. These results suggest that Dimera0 and Dimera3 are equally specific for the HER-2 mRNA molecule, despite their difference is spacer lengths.

Trimeric antisense compounds do not markedly enhance RNaseH mediated cleavage of the 5'-UTR of the HER-2 mRNA over that of the best dimeric compound.

Proving that an antisense molecule, chimeric in nature, designed to specifically target the single stranded regions of an RNA molecule can enhance antisense potency, we continued with this approach by adding a third hexamer to our dimeric compound. Based on data derived in Figure 8, we determined that the most accessible region in the 5'-UTR of the HER-2 mRNA was labeled as loop 8b. We chose the hexamer complementary to loop 8b to serve as a second "binding" domain. The hexamer consisted of 2'-O-methyl RNA nucleotides, which have been shown to bind to target RNA with great affinity and exhibit nuclease resistance but do not recruit RNaseH. The third hexamer was attached to Dimera3-PS at the 3' end of the phosphorothioate hexamer and to the 5' end of the third hexamer. The resulting trimeric molecule used consists of three hexameric oligonucleotides chemically linked to each other and oriented in a 5' to 3' direction. (Figure 10) The 5' hexamer of the trimeric molecule consists of a 2'-O-methyl RNA hexamer complementary to loop3 in the HER-2 mRNA, chemically linked in a 5' to 3' orientation by 3 9-carbon spacers to a second phosphorothioate hexamer which is complementary to loop 2. The second hexamer is chemically linked to the 3'end hexamer consisting of 2'-O-methyl RNA complementary to loop 8b in a 5' to 3' orientation.

In order to determine whether these novel antisense compounds would promote RNaseH mediated cleavage of the target HER2 mRNA, trimeric antisense compounds, labeled Trimera0-PS through Trimera3-PS, were constructed with different multimer 9-carbon spacers linking the central hexamer and the 3' end hexamer. The 5'-[32P]-labeled HER-2 mRNA was subjected to RNaseH digestion in the presence of the trimeric molecules, and the extent of cleavage of HER-2 mRNA was compared to reactions containing equal concentrations of both Dimera3 and the PS-hexamer complementary to loop 2. The results in Figure 15 show that Trimera0-PS slightly increases the sensitization of HER-2 mRNA to RNaseH activity at a concentration of 0.1μM compared to Dimera3-PS and Loop 2-PS, as indicated by production of the cleaved RNA fragment and by disappearance of the precursor RNA. Trimeric compounds labeled 1 through 3 did not show the ability to increase the sensitization of HER-2 mRNA to RNaseH activity at 0.1μM as compared to Dimera3-PS and were less stimulatory than Trimera0-PS.

Next we evaluated the specificity of the trimeric antisense compounds by performing RNaseH sensitization assays in the presence of both 1 and 5 μg/ul of total yeast RNA. Figure 16 shows the ability of the trimeric compounds, Dimera3-PS, and Loop 2-PS at 0.1μM to promote RNaseH cleavage. The results suggest that the trimeric compounds do not increase their specificity for the HER-2 mRNA target as compared to either Dimera3-PS or Loop2-PS.

Dimera3-PS, but not Dimera0-PS, inhibits HER-2/luciferase expression in T7 coupled wheat germ extracts

The RNaseH sensitization assays show that we can construct antisense compounds that can stimulate RNaseH mediated cleavage of the 5' UTR of HER2 mRNA. Our next step is to show that these antisense compounds can inhibit the expression of HER2. While the RNaseH assays yielded encouraging results, it is important to evaluate the antisense compounds in their ability to inhibit protein expression. Several cell-free translation assays have been used to show that antisense compounds can down express a target gene (Tinevez et al., 1998). In this report, we have constructed a vector containing the 5'-UTR of the HER-2 gene contiguous with the luciferase reporter gene in a pBluescript vector driven by a T7 promoter, labeled T7/HER-2/Luciferase (the T7/luciferase plasmid, T7/Luciferase, was kindly donated by Dr. Peltz, UMDNJ). A graphic representation of the constructs is shown in figure 17.

We used a T7 RNA polymerase wheat germ cell-free assay (Promega) in which the T7/HER-2/Luciferase construct was added to the extract in the presence of our novel chimeric antisense compounds. We tested our most potent dimeric compounds, as determined by the RNaseH sensitization assays, for the ability to inhibit the expression of luciferase in both the T7/HER-2/Luciferase vector and the control vector. The DNA

hexamer complementary to loop 2 for the HER-2 mRNA was also tested. The DNA hexamer and the dimeric antisense compound, Dimera3, did not inhibit expression of luciferase in T7 wheat germ extracts at concentrations as great as 10μM, suggesting that the DNA component of the antisense molecule is sensitive to nucleases present in the extract (Figure 18). The phosphorothioate modified hexamers, either alone or as part of the chimeric antisense compound, inhibited expression of luciferase in the T7/HER-2/Luciferase construct at 10μM. (Figure 19) Dimera0-PS inhibited luciferase activity at both 5μM and 10μM. Phosphorothioate modified hexamer complementary to loop 2 of the HER-2 mRNA was shown to inhibit luciferase expression in the T7/HER-2/Luciferase construct at 10μM. It is most likely, based on the RNaseH sensitization assays, the inhibition seen by both Loop 2-PS and Dimera0-PS are due to non-antisense effects. Further evidence confirming the non-antisense effects of the phosphorothioate modified antisense compounds can be gleaned by the inhibition of luciferase activity in wheat germ extracts using scrambled antisense compounds. Scrambled antisense compounds, (Scrambled Dimera0-PS, UGCGGU(2'-O-methyl RNA)CGCCGG(PS)), and Scrambled Dimera3-PS, UGCGGU(2'-O-methyl RNA)C9/C9/C9/CGCCGG(PS)) were constructed by rearranging the sequence order of both hexamers of the dimeric compounds while maintaining the same nucleotide base content. Scrambled Dimera0-PS and Scrambled Dimera3-PS inhibited luciferase expression at 10μM in both the T7/HER-2/Luciferase construct, as shown in Figure 22. The result differs from the RNaseH sensitization assays, whereby the scrambled dimeric antisense compounds failed to promote RNaseH mediated cleavage of the 5'UTR of the HER-2 mRNA at a concentration of 1μM (Figure 13). This inhibition is likely due to non-antisense effects and not due to specific sequence interaction with another part of the 5'-UTR HER-2 mRNA. Indeed, one of the drawbacks of using phosphorothioate modified antisense oligonucleotides is the non-antisense effects that have been reported when used in vivo. (Agrawal et al, 1997,and Brukner et al, 2000)

The trimeric antisense compounds stimulate expression of luciferase activity in wheat germ cell free extracts

We then tested for the ability of the most potent trimeric antisense molecule, Trimera0-PS, to down-regulate luciferase activity in T7 wheat germ extracts containing either the control vector, lacking the HER-2 leader, or the T7/HER-2/Luciferase vector containing the HER-2 leader sequence. Antisense compounds Dimera0-PS, Dimera3-PS, and Trimera0-PS all decreased luciferase activity in the control vector at 10μM, but not at or below 1μM. This effect is believed to be non-specific inhibition of translation. (Figure 20) Dimera0-PS and Dimera3-PS inhibited luciferase activity at 10μM, while Dimera3-PS was able to significantly inhibit the expression of luciferase at 1μM (50% inhibition) and slightly at 0.1μM (20% inhibition) (Figure 19 and 21). Trimera0-PS however, shows a significant stimulation at 1μM (10-fold) and that stimulation was still present at 10μM (Figure 21). These results were unexpected. This experiment was repeated twice, both yielded the same results. (data not shown)

To determine whether this phenomenon was restricted to only Trimera0-PS or was common among the other trimeric compounds, Trimeras0-PS through Trimera3-PS were tested for their ability to inhibit expression of luciferase in wheat germ extract. Our results show that all the trimeric compounds tested stimulated luciferase expression at 1μM, but Trimera0-PS was the most potent stimulator of luciferase expression. (Table 4) This phenomenon is dependent on the HER-2 leader sequence since Trimera0-PS fails to stimulate luciferase expression at 1uM in the control T7/luciferase vector. We are currently following up these interesting results by testing whether Trimera0-PS can similarly stimulate HER-2 expression in breast cancer cells that are low HER-2 expressers.

Statement of Work Evaluation:

As pointed out in the review of May 25, 2001 from Ms. Pawlus, Tasks 1 through 6 has been completed. The remaining Task 7 concerns testing our best antisense agent/conjugate in a HER-2/nue overexpressing breast cancer cell line. Actually, we substituted another task for Task 7. This intermediate task uses a cell-free (wheat germ) extract to avoid the complication of uptake into cells and thereby focus on the direct effect of our antisense agent on the target 5'-UTR of HER-2 mRNA. The cell free extract uses a plasmid in which our target, the 5'-UTR of HER-2 mRNA is fused to a luciferase reporter gene. When we tested with our dimeras, luciferase was inhibited, as expected. When tested with our trimeras, unexpectedly, luciferase was stimulated even though the RNaseH assay indicated degradation of the 5'-UTR. Since this finding could be a clue as to the mechanism for HER-2 overexpression in certain breast cancers, we must confirm this seemingly contradictory observation in breast cancer cells. Thus, we have completed the substituted Task 7, but we find it necessary to pursue the original Task 7.

Task 7 concerns stimulating degradation of HER-2 mRNA by RNaseH in whole cells. However, the problem of getting antisense DNA into whole cells, particularly into the cytosol rather than sequestered inside lysosomes, outweighs the problem of getting a strong and specific stimulator of RNaseH. In separate studies on peptides which, like antisense DNA, require access to targets in the cytosol, we have achieved enhanced uptake into cells while avoiding lysosomal entrapment. We use a non-toxic polymer, poly(ethylene glycol) (PEG), which has, for example, 8 attachment sites. In our peptide studies, we attached to each site a copy of the Tat basic domain peptide, which has 8 positive charges and to which is appended a molecule of the vitamin, biotin (Ramanathan et al.). Uptake of this conjugate, in which the peptide serves both as the uptake enhancer and as the therapeutic agent, is efficient and avoids lysosomal entrapment. A US patent (#6,258,774) has just been issued to S. Stein, M. J. Leibowitz and P. J. Sinko having claims for intracellular delivery of therapeutic agents, including antisense DNA.

We now plan to synthesize an antisense agent based on our best RNaseH stimulating oligonucleotide. At the 5' terminus will be appended a Tat analog, the peptide $dArg_8dLys$(biotin). At the 3' terminus will be a thiol group to form a disulfide bond with the polymer. The final conjugate will be:

branched PEG-[disulfide bond-3'-antisense DNA chimera5'-d-Arg_8dLys(biotin)]$_8$.

It will be tested on several HER-2 overexpressing cell lines using flow cytometry to quantitate cell surface expression of the HER-2/neu protein.

For preclinical testing, which will be the subject of a future grant application, this conjugate will be injected IV or IM. It will tend to accumulate in tumors due to its high molecular weight and extended shape. It will bind to cell surfaces due to the high positive charge of the peptides (irrespective of the slightly higher number of negative charges on the DNA). It will lock into the biotin transporter protein (also known as the sodium dependent multivitamin transporter). However, instead of endocytotic uptake, the PEG will fuse with the cell membrane (Sinko, et al., unpublished results), and the entire conjugate will enter the cytosol. The disulfide bonds will be cleaved by intracellular glutathione reduction, releasing the individual antisense DNA-peptide (biotin) molecules. The antisense DNA will bind its complementary sequence in the HER-2 mRNA with the peptide serving a second function, to increase binding avidity, and hence potency, by an order of magnitude (Wei et al., 1996). RNaseH will degrade the target mRNA, releasing the conjugate, which should be highly resistant to degradation, to act in a catalytic fashion. Furthermore, this PEG conjugate may also have oral bioavailability, making it convenient to be taken as a pill rather than as an injectable drug. We plan to continue development of this novel therapeutic approach.

Key Research Accomplishments:

1. Production of a synthetic cyclic peptide that binds specifically to HER-2/neu mRNA

2. Production of short antisense oligonucleotides that sensitize HER-2/neu to enzymatic cleavage by RNaseH

3. Production of chimeric oligonucleotides that greatly enhance sensitization of HER-2/neu mRNA to enzymatic cleavage by RnaseH

Note: A patent application is in preparation on the chimeric antisense agents.

Reportable Outcomes:

1. Abstract Presentation - Poster Assignment: CC-38

   Era of Hope
   Department of Defense Breast Cancer Research Program Meeting
   Atlanta Georgia

   Abstract Title: DUAL-SPECIFICITY ANTI-HER-2/NEU ANTISENSE DNA AGENTS FOR BREAST CANCER THERAPY

   Authors: M. J. Leibowitz, J. Perlman, Q. Ding and S. Stein

2. Abstract Presentation

   7th RCMI International Symposium Abstract Form

   Abstract Title: BI-FUNCTIONAL ANTISENSE AGENTS AGAINST HER-2/neu
   November 2000, Puerto Rico

   Authors: M.J. Leibowitz, J Perlman, Q. Ding, S. Pooyan, and S. Stein

3. Papers

   S. Ramanathan, B. Qui, S. Pooyan, S. Stein, M.J. Leibowitz and P.J. Sinko. Targeted PEG-based bioconjugates enhance the dellular uptake and transport of an HIV-1 Tat nonpeptide. *J. of Controlled Release*, in press (2001)

4. Patent

   S. Stein, M.J. Leibowitz and P.J. Sinko. Carrier for in vitro delivery of a therapeutic agent, U.S> Patent 6,258,774 (July 10,2001).

5. Degrees None.

Conclusions:

Sequence analysis and comparison of the 5' untranslated region of the HER-2/neu gene revealed a discrepancy among previously published sequences. This discrepancy could be explained in several ways. The different published sequences might be the result of a genetic rearrangement in the 5'-untranslated region of the HER-2 gene, and such genetic rearrangements are common among various cancer cells. It is also possible that this region represents a highly polymorphic region of the human genome. Further PCR experiments applying the various upstream primers to a series of different cancer cell lines were performed. In all cancer lines tested, the primers that amplified the 5' untranslated region of the HER-2 gene were those that were published in Child et al, 1999. The sequenced PCR product matched the HER-2 sequence used by Child et al. and was the sequence we used in our RNaseH and wheat germ assays. These results fail to determine the origin of the divergent sequences reported. We also attempted to identify other regions of the human genome from which translocated DNA might have been brought into proximity with the HER-2 gene via recombinational events using nucleotide BLAST searching, and have failed to find such sequences.

Through combinatorial chemistry, we have been able to construct synthetic peptides that vary significantly in their binding to HER-2 mRNA. Further deconvolution should produce peptides that will be able to selectively bind to HER-2 mRNA with greater affinity. However, our initial bifunctional antisense molecules were made by another strategy, not utilizing peptides. Indeed, we have focused instead on this new approach in designing novel antisense molecules.

We were able to produce short antisense oligonucleotides that are able to sensitize HER-2 mRNA to enzymatic cleavage by RNaseH. These short hexameric oligonucleotides take advantage of the unique 3-dimensional structure of RNA molecules and are targeted to single-stranded regions of the HER-2 mRNA. We were able to combine the DNA hexamers that are complementary to the most accessible sites on the 5'-UTR of the HER-2 mRNA, based on the RNaseH sensitization assays, together to create dimeric and trimeric antisense compounds that specifically target the HER-2 mRNA and stimulate RNaseH mediated cleavage of the HER-2 mRNA. The construction of the dimeric antisense molecules includes a 2'-O-methyl RNA hexamer which serves as the "anchor", and is complementary to loop 3 in the HER-2 mRNA molecule, and a DNA hexamer which serves as the "active" site for RNaseH action, complementary to loop 2 of the HER-2 mRNA. The dimeric antisense compounds were shown to significantly increase the sensitivity of the 5'-untranslated region of the HER-2 mRNA over the hexamers alone. The effectiveness of these dimeric antisense molecules are dependent on the length of the spacer between the two hexamers, suggesting that antisense molecules can be produced to target the accessible single-stranded regions of a particular RNA. We confirmed that phosphorothioate modified antisense molecules are not as potent RNaseH stimulators than DNA, as determined by our RNaseH sensitization assays.

This approach to antisense design is powerful in that it utilizes existing single-stranded regions in the HER-2 mRNA molecule, reducing the binding penalties associated with antisense invasion of double-stranded regions of a RNA molecule. We have also been able to show that the dimeric antisense molecules are effective specifically down-regulating a luciferase reporter gene with the HER-2 leader sequence directly upstream of the luciferase ORF. We tested our best dimeric antisense compounds in couple T7 polymerase wheat germ extracts, using the T7/HER-2/luciferase construct. Our studies showed that the dimeric compounds with an "active" site consisting of DNA failed to inhibit expression of luciferase in the T7/HER-2/luciferase construct, suggesting that these antisense compounds were degraded in the wheat germ extract by nucleases. The dimeric antisense compounds with phosphorothioate modified "active" sites, while shown to be less potent in their ability to stimulate RNaseH cleavage in our sensitization assays, showed greater effectiveness in the T7 coupled wheat germ assays. Dimera0-PS failed to specifically inhibit the T7/HER-2/luciferase construct, but Dimera3-PS did show specific inhibition as low as 0.1μM.

We continued this approach to antisense construction by creating trimeric antisense compounds. The production of the trimeric compound was based on one of our best dimeric compounds, Dimera3-PS. We chose

a third hexamer, complemetary to the 5'-UTR of the HER-2 mRNA and highly stimulated RNaseH mediated cleavage, to serve as a second "anchor" site. This hexamer consists of 2'-O-methyl RNA and was attached to the dimeric compound by different linked 9-carbon spacers at the 3' end of the dimeric antisense compound. The trimeric compounds did not enhance stimulation of RNaseH mediated cleavage of HER-2 mRNA, and were approximately equivalent to the dimeric compounds.

We tested our trimeric antisense compounds in the T7 coupled wheat germ cell free extract. The trimeric antisense compounds were found to significantly stimulate expression of luciferase in the T7/HER-2/luciferase construct at 1μM and 10μM. Trimera0-PS was the potent stimulator, increasing luciferase expression 10-fold. However, all trimeric antisense compounds tested increased expression of the T7/HER-2/luciferase construct at 1μM. The trimeric compounds did not stimulate luciferase expression in the T7/luciferase control vector, indicating that this effect is specific for the HER-2 leader.

This finding was unexpected and has prompted us to examine the potential mechanisms underlying this phenomenon. Indeed, the 5' UTR of many genes play a role in translational regulation (Fiaschi et al., 2000). Specifically, two characteristics are present in the 5'-UTR of theHER-2 ORF, or that a play a critical role in translation, a stable secondary structure and an upstream uORF (Fiaschi et al., 2000). Several reports have shown that an upstream uORF in the 5'-UTR of an mRNA can repress translation and that upstream open reading frames are enriched in certain gene families, such as proto-oncogenes (Kwon et al., 2001), of which HER-2 is one. It has now been shown that the HER-2 uORF does repress downstream translation in several cell types (Child et al., 1999), indicating its regulatory activity.

It has also been shown that the secondary structure of the 5'-UTR of the muscle acylphosphatase mRNA is involved in the regulation of protein expression (Fiaschi et al., 2000). We hypothesize that the trimeric antisense compound is acting on the 5'-UTR of the HER-2 mRNA, resulting in an increase in expression of the luciferase protein. We are now testing HER-2 overexpressing and low HER-2 expressing breast cancer cell lines in order to determine whether we can show the stimulatory effect in cells. We will also test whether on HER-2 overexpressing breast cancer cells might stimulate expression of this gene by a similar mechanism.

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Captions:

Table 1. Binding results of the first deconvolution with cyclic peptides on SPOTs membrane. Positions X1 and Y1 were deconvoluted using synthetic amino acids shown In Fig. 2. The amino acids listed along the top of the table refer to amino acids in the Y1 position and amino acids listed down the table refer to amino acids in the X1 position. The other positions were held constant and contained D-Arg. Binding assays were conducted in PBS at 37°C for 2 hours. The spots on the membrane were measured for HER-2 binding and expressed as percent of the HER-2 mRNA bound. The peptide exhibiting the strongest affinity ofr HER-2 contained ala-3-pyr in the X1 position and homo-Lys in the Y1 position. These two positions were fixed during the second deconvolution.

Table 2. Binding results of the second deconvolution with cyclic peptides. Positions X1 and Y1 were fixed with the amino acids selected as described in Table 1 while positions X2 and Y2 varied. Binding assay conditions were the same as the earlier binding assay.

Table 3. Binding results of the third deconvolution with cyclic peptides. Positions X1,X2,Y1, and Y2 were fixed as described in Tables 1 and 2, while positions X3 and Y3 varied.

Figure 1. Schematic representation of the cyclic peptides. As indicated in the text and Tables 1-3, cyclic peptides were deconvoluted at the positions labeled X1 through X3 and Y1 through Y3. Construction of cyclic peptides was performed on the SPOTs membrane.

Figure 2. Schematic representation of the synthetic amino acids used in the deconvolution process of reiterative synthesis and screening of cyclic peptides binding to HER-2 mRNA.

Figure 3. Schematic representation of the cyclic peptide exhibiting the strongest affinity for HER-2 mRNA.

Figure 4. Comparison of the reported HER-2 DNA sequences in the 5' untranslated region. Here capital letters indicated sequences identical with the sequence described by child et al. (1999), and lower cases letters indicate sequences diverging from that sequence. Solid arrows indicate primers that resulted in successful PCR amplification from normal human genomic and breast cancer cell line DNA. Dashed arrows indicate primers that did not yield PCR products. PCR was performed with a conserved downstream primer derived from the ORF in exon 1, which was identical in all reported sequences.

Figure 5. Structure of the 5' untranslated region of the HER-2 mRNA. The 165 base sequence was subjected to the Wisconsin GCG software RNA mfold program. Schematic representation of the folded structure shown in the figure is based on predicted

energy levels of base pairing to generate this structure. DNA hexamers were constructed complmentary to the possible loops and stem regions, highlighted in yellow.

Figure 6. RNaseH sensitive regions of the 5' region HER-2 mRNA. DNA hexamers complementary to the possible loop2 and a stem region in the HER-2 mRNA structure (see Fig. 5) were hybridized to the 5' labeled RNA, which was subjected to digestion with RNaseH and analyzed by polyacrylamide gel electrophoresis in the presence of 8M urea. Untreated RNA and RNA treated with RNaseH in the absence of oligonucleotide are also indicated.

Figure 7. Comparison of the affinity of antisense hexamers for RNA. The experimental procedure was as described in Fig. 6, except the indicated lower concentrations of antisense hexamers were used.

Figure 8. RNaseH sensitive regions of the 5' region HER-2 mRNA. All DNA hexamers complementary to the possible loop regions in the HER-2 mRNA structure (see Fig. 5) were hybridized to the 5' labeled RNA, which was subjected to digestion with 2 units of RNaseH and analyzed by polyacrylamide gel electrophoresis in the presence of 8M urea. Hexamers effective at promoting RNaseH cleavage at 10μM result in the disappearance of the precursor RNA and the appearance of degraded products.

Figure 9. The most accessible single-stranded regions to RNaseH of the 5' region HER-2 mRNA. A random hexamer library was hybridized at increasing concentrations to the 5' labeled RNA, which was then subjected to digestion with 2 units of RNaseH and analyzed by polyacrylamide gel electrophoresis in the presence of 8M urea. DNA hexamers complementary to loop 2, 3, and 8b of the HER-2 mRNA were used to compare the most sensitive sites derived by the random hexamer library to the sites directed by the RNA folding program.

Figure 10. A schematic representation of the construction of the chimeric antisense molecules. The trimeric compound is constructed based on the most potent dimeric antisense compound.

Figure 11. Enhanced sensitivity of 5' region of mRNA to RNaseH by antisense chimeric dimers. The experiment is as described in Fig. 6. Dimera 1 has a single 9-carbon linker and Dimera 3 has 3 9-carbon linkers between the "active" and "binding" hexamers.

Figure 12. The comparison of different dimeras for RNaseH sensitization of HER-2 mRNA. The procedures were as in Fig. 6, where Dimeras 0,1,2,3, and 4 have the corresponding number of 9-carbon linkers connecting the "active" and "binding" hexamers.

Figure 13. Comparing the ability of phosphorothioate modified vs. DNA hexamer dimeric antisense compounds to promote RNaseH mediated cleavage of the 5'-UTR HER-2 mRNA. The procedures were as described in figure 6. Dimeric compounds containing the "active" site constructed of DNA exhibited a stronger ability to sensitize

the HER-2 mRNA to RNaseH cleavage at 0.1μM and 0.01μM than that of dimeric compounds that have a phosphorothioate modified "active" site.

Figure 14. The dimeric compounds Dimera0 and Dimera3 as well as the hexamer complementary to loop2 of the HER-2 mRNA were evaluated for their ability to specifically promote RNaseH mediated cleavage in the presence of total yeast RNA. The procedure is as previously described in figure 6.

Figure 15. Comparing the ability of trimeric antisense compounds to promote RNaseH mediated cleavage of the 5'-UTR HER-2 mRNA. The trimeric compounds do not enhance RNaseH cleavage of the HER-2 mRNA as compared to Dimera3-PS at concentrations of 0.1μM, 0.01μM, and 0.001μM. Scrambled dimeric antisense compounds failed to promote RNaseH cleavage at 1μM. Procedure is as previously described in figure 6.

Figure 16. The comparison of the trimeric antisense compounds to the dimeric compounds in their ability to promote RNaseH mediated cleavage of the 5'-UTR HER-2 mRNA in the presence of total yeast RNA. The trimeric compounds do not show enhanced specificity to the HER-2 mRNA as compared to the dimeric antisense compounds. Procedure is as previously described.

Figure 17. A schematic representation of the construction of the T7/HER-2/luciferase plasmid used in the T7 coupled wheat germ extract. The T7/luciferase construct was co-transformed with a PCR amplified fragment of the 5'-untranslated region of the HER-2 gene in DH5α cells. The resulting construct contains the HER-2 leader sequence directly upstream of the luciferase reporter gene driven by a T7 promoter.

Figure 18. The effects of Dimera-3-DNA on the expression of the T7/HER-2/luciferase in wheat germ extract. 1μg of the T7/HER-2/luciferase vector was added to wheat germ extract in the presence of increasing concentrations of the Dimera-3-DNA antisense compound. The wheat germ extract was incubated at 37°C for 90 minutes, and luciferase activity was measured via a luminometer. No effect on expression was observed at concentrations as high as 10μM.

Figure 19. The effects of Dimera0-PS and Dimera3-PS on expression of the T7/HER-2/luciferase in wheat germ extract. Dimera3-PS inhibited luciferase expression by 50% at 1μM and by 20% at 0.1μM, while Dimera0-PS fails to inhibit expression of luciferase at either 1μM or at 0.1μM. The procedure is as previously described in figure 18.

Figure 20. The effects of both the dimeric and trimeric antisense compounds on expression of the T7/luciferase control vector in wheat germ extract. All the compounds tested inhibited expression of luciferase at 10μM, but not at 1μM or at 0.1μM. Procedure is as previously described in figure 18.

Figure 21. The effects of both the dimeric and trimeric antisense compounds on expression of the T7/HER-2/luciferase construct in wheat germ extract. Trimeria0-PS

stimulated expression of luciferase at 1μM and at 10μM. Dimera3-PS inhibited luciferase expression by 50% at 1μM and by 20% at 0.1μM, while Dimera0-PS fails to inhibit expression of luciferase at either 1μM or at 0.1μM. The procedure is as previously described in figure 18.

Table 4. The effects of the trimeric compounds on expression of the T7/HER-2/luciferase in wheat germ extract at 1μM. All the trimeric compounds stimulated expression of luciferase at 1μM. Trimera0-PS was the most potent stimulator of luciferase expression, increasing expression 10-fold. The conditions of the assay were carried out as previously described in figure 18.

Figure 22. The effect the scrambled antisense compounds and a phosphorothioate-modified hexamer on expression of the T7/HER-2/luciferase construct in wheat germ extract. Both scrambled antisense compounds as well as the PS-modified hexamer complementary to loop 2 of the 5'-UTR HER-2 mRNA inhibited luciferase expression at 10μM. The conditions of this assay were carried out as previously described in figure 18.

Peptide Library 1

X \ Y	D-Lys	2-ABZ-OH	Homo-Lys	Ala-4-pip	L-m-Tyr	L-Tyr(Bn, 3-Cl)	Gly-4-pip	Ala-3-pyr
D-Lys	1.4	0.7	1.6	1.7	1.7	1.5	1.4	0.7
2-ABZ-OH	1.3	0.4	1.5	1.4	1.4	1.4	1.7	1.1
Homo-Lys	1.7	0.9	1.4	1.5	1.5	1.3	1.5	1.3
Ala-4-pip	1.7	0.7	1.2	1.3	1.3	2.1	2.2	1.4
L-m-Tyr	1.8	0.6	1.4	1.5	1.2	1.7	1.9	1.8
LTyr(Bn, 3-Cl)	2.5	1	1.8	2.2	1.7	0.9	2.3	1.8
Gly-4-pip	1.7	1.1	2	1.6	1.6	2.3	2	1.6
Ala-3-pyr	1.8	1	**2.6**	2.9	2.2	2.1	2.2	1.6

Selected: X_1=Ala-3-pyr
Y_1=Homo-Lys

Table 1

Peptide Library 2

X \ Y	D-Lys	2-ABZ-OH	Homo-Lys	Ala-4-pip	L-m-Tyr	L-Tyr(Bn, 3-Cl)	Gly-4-pip	Ala-3-pyr
D-Lys	1.3	1.1	1	1.1	0.9	1.8	2.1	2
2-ABZ-OH	1.7	1.2	1.7	1.3	1	1.2	1.4	1.6
Homo-Lys	1.9	1.7	2	2	2.1	2.2	1.8	2.2
Ala-4-pip	2	1.8	1.9	2	2.1	1.9	1.9	2.3
L-m-Tyr	1	0.5	0.5	0.7	1.3	1.4	2	1.7
L-Tyr(Bn, 3-Cl)	0.6	0.6	0.7	0.7	0.9	1.5	1.5	1.4
Gly-4-pip	2.2	2.1	2.4	**2.9**	2.7	1.8	1.9	2
Ala-3-pyr	1.8	1.6	1.6	1.9	1.8	1.4	1.5	1.4

Selected: X2= gly-4-pip
Y2=Ala-4-pip

Table 2

Library 3

X/Y	D-Lys	2-ABZ-OH	Homo-Lys	Ala-4-pip	L-m-Tyr	L-Tyr(Bn, 3-Cl)	Gly-4-pip	Ala-3-pyr
D-Lys	1.1	1	2.4	**3**	1.9	2.7	2.9	3.2
2-ABZ-OH	1	0.5	1.6	1.8	0.6	1.5	1.6	2
hLys	0.9	0.9	2	3	2.1	2.6	2.2	2.9
Ala-4-pip	1	1.1	1.7	2.7	1.8	1.6	2.3	**3**
L-m-Tyr	0.4	0.2	0.8	1	0.4	0.3	0.5	0.7
L-Tyr(Bn, 3-Cl)	0.6	0.7	0.4	1.2	0.4	0	0.3	0.1
Gly-4-pip	2.2	1.4	1.7	1.6	1	1	1.4	1
Ala-3-pyr	2.1	1.6	1.9	1.5	0.9	1.6	2.1	1.3

Selected: X3=D-Lys
Y3=Ala-4-pip

Table 3

STRUCTURE OF STARTING PEPTIDE

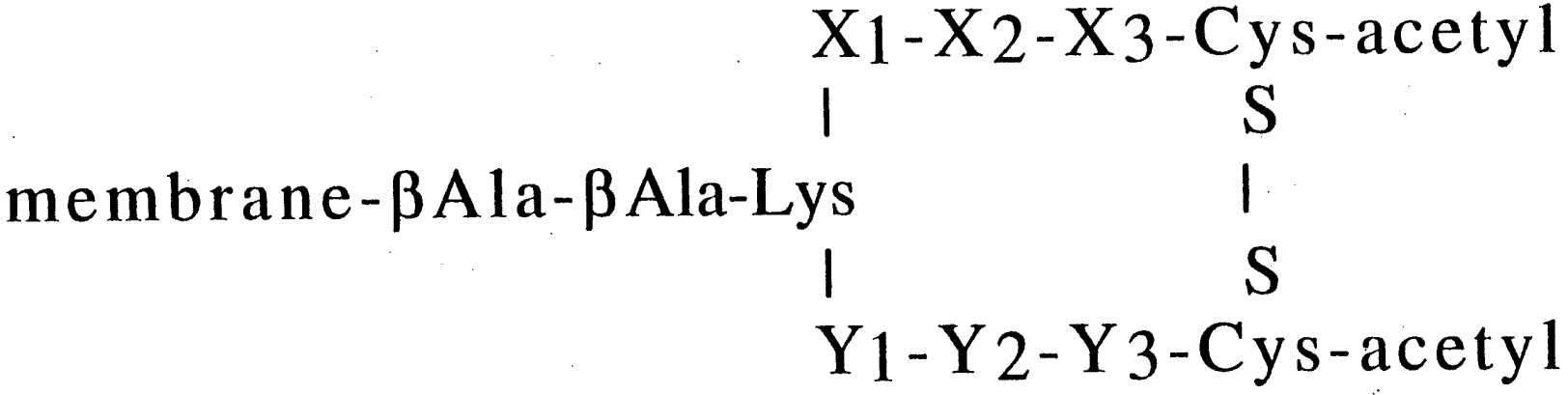
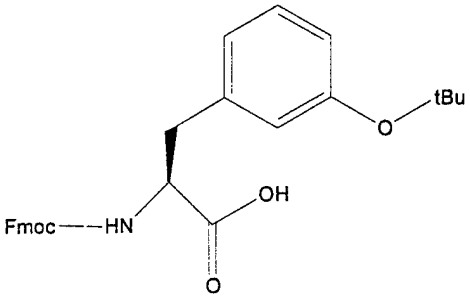
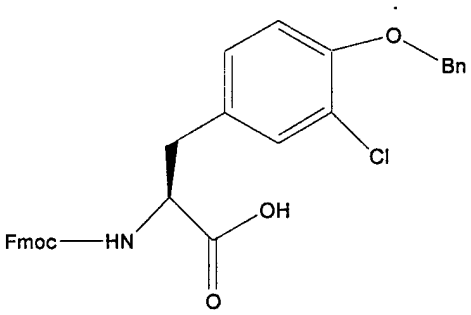


Figure 1

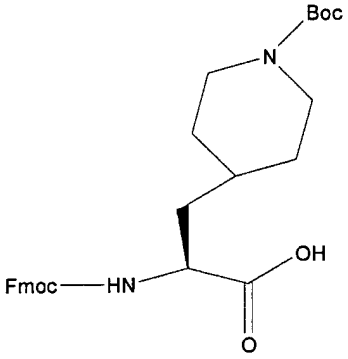
Amino Acid Analogues for Synthesis of Peptide Library



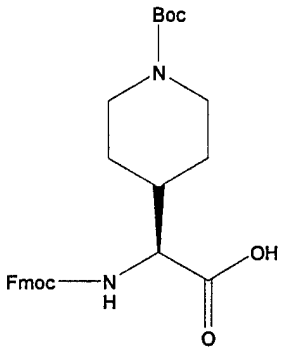
Fmoc-L-m-Tyr(tBu)



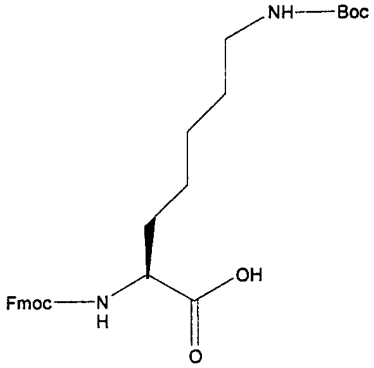
Fmoc-L-Tyr(Bn, 3-Cl)



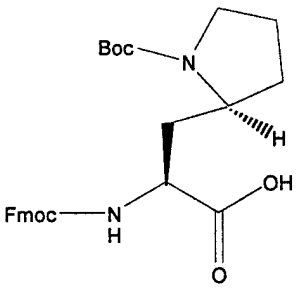
Fmoc-L-Ala-4Pip(Boc)



Fmoc-L-Gly-4Pip(Boc)



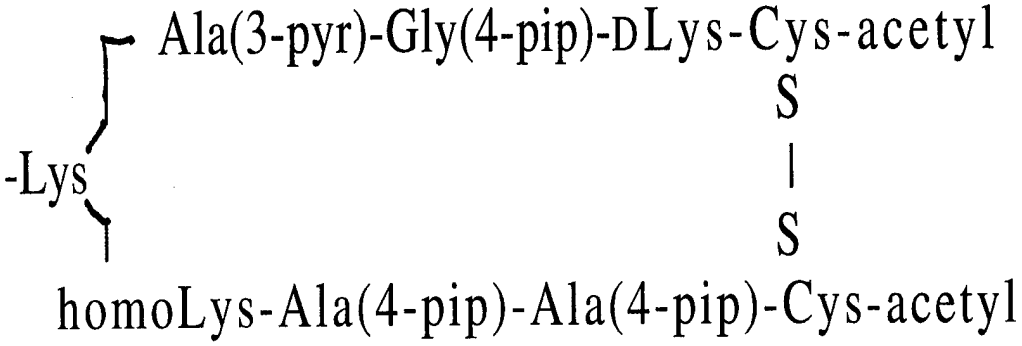
Fmoc-L-hLys(Boc)



Fmoc-L-Ala(3-pyrolidinyl-(2-N-Boc)

Figure 2

STRUCTURE OF BEST PEPTIDE



The conformationally constrained cyclic peptide of the above design was determined to be the strongest binder of radiolabeled HER-2 RNA in the presence of a large background of total yeast RNA. A second peptide with nearly as strong binding had m-Tyr at position Y2. Deconvolution cycles may be repeated at each position using the same or additional amino acids. The next step in this project would be to append a HER-2 antisense DNA to this best peptide and test for inhibition of HER-2 expression.

Figure 3

HER2/neu Sequence Comparison of 5' Untranslated End

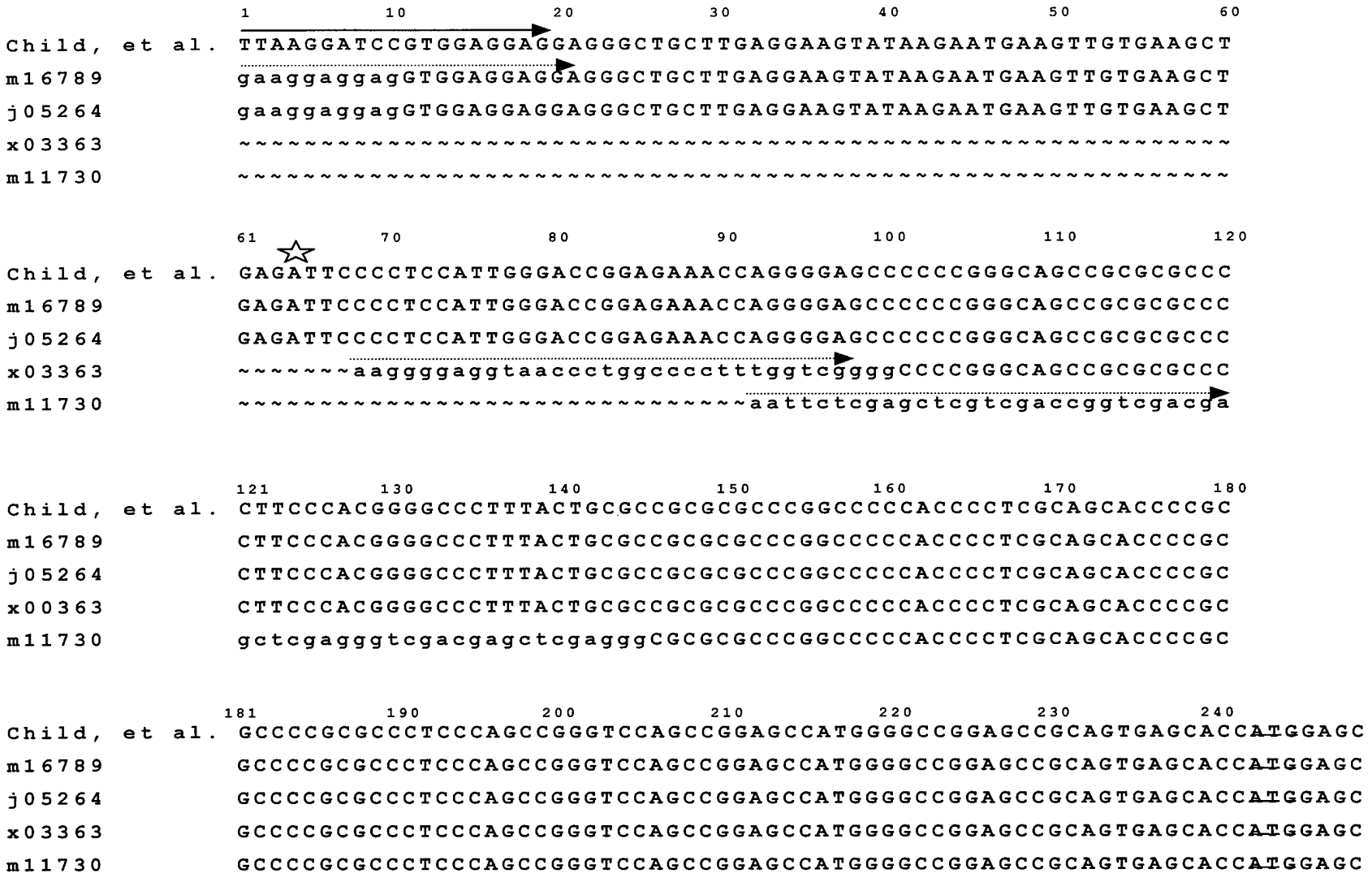


Figure 4

Targets of Antisense Hexamers on 5' Untranslated Region of HER2/neu mRNA

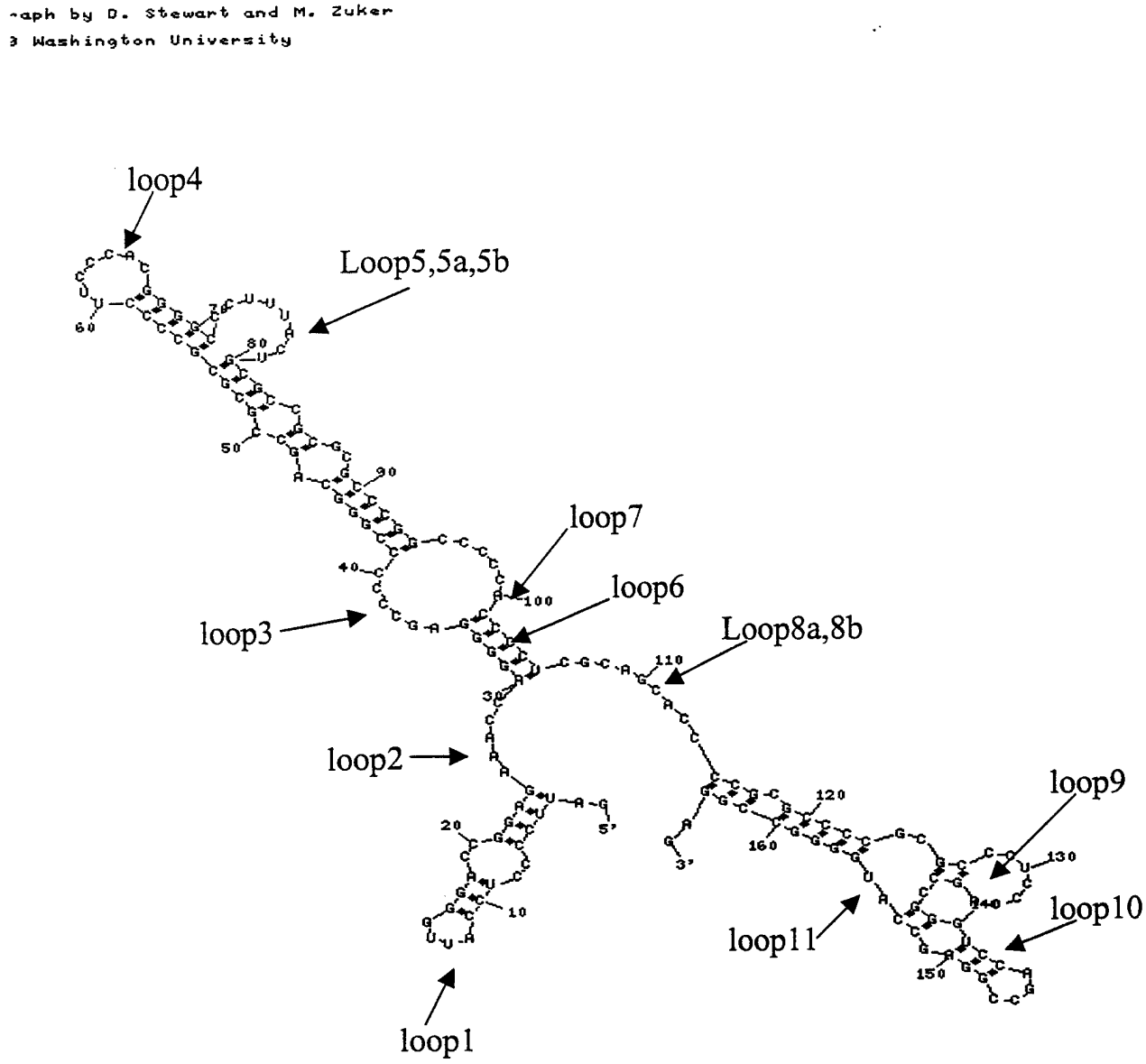


Figure 5

Comparison of RNaseH Sensitization of 5' Region of mRNA by Antisense Hexamers

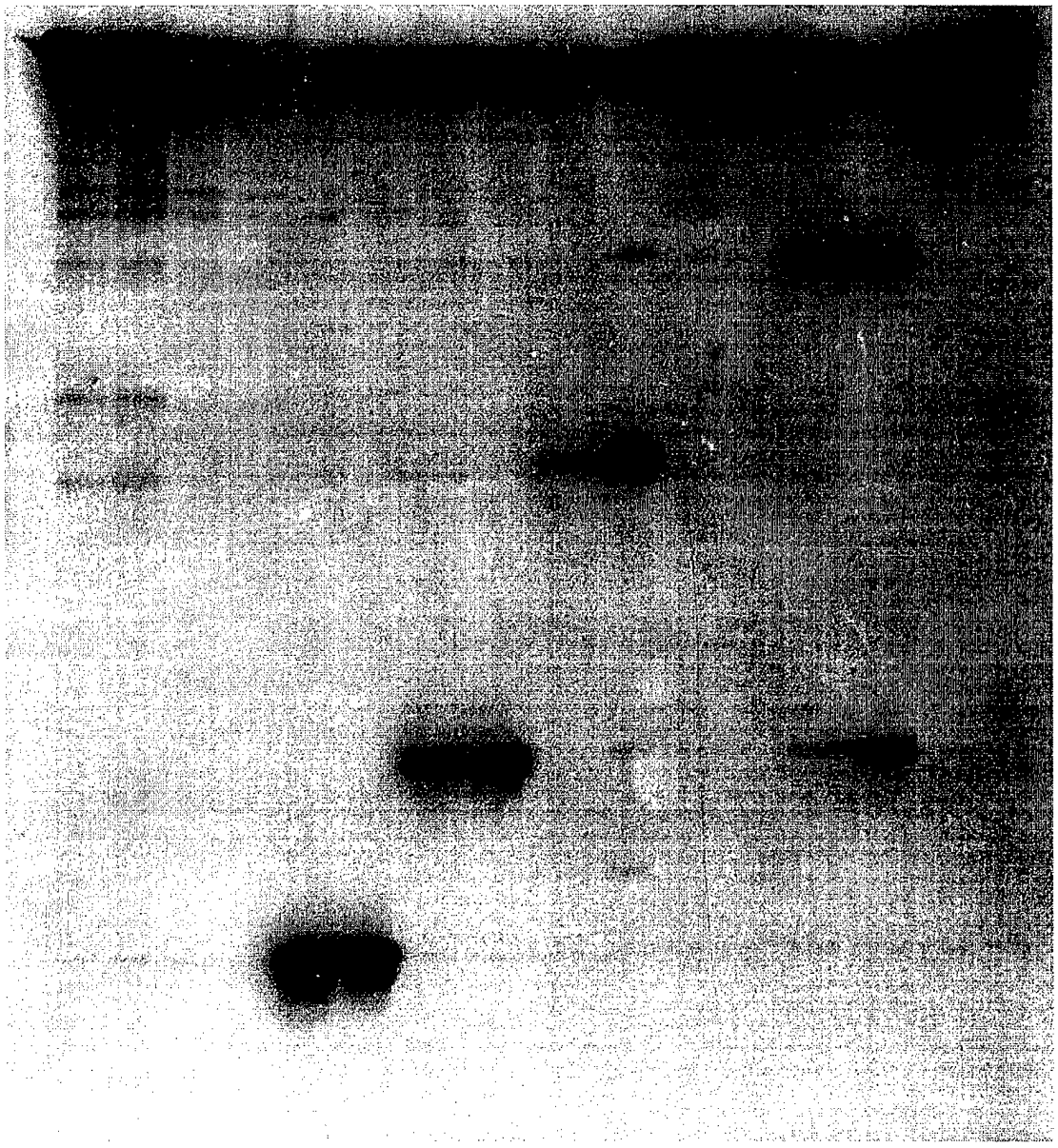


Figure 6

Loop 3 is the Most Sensitive Target for Antisense Binding

Figure 7

Comparison of RNaseH Sensitization of 5' Region of mRNA by Antisense Hexamers

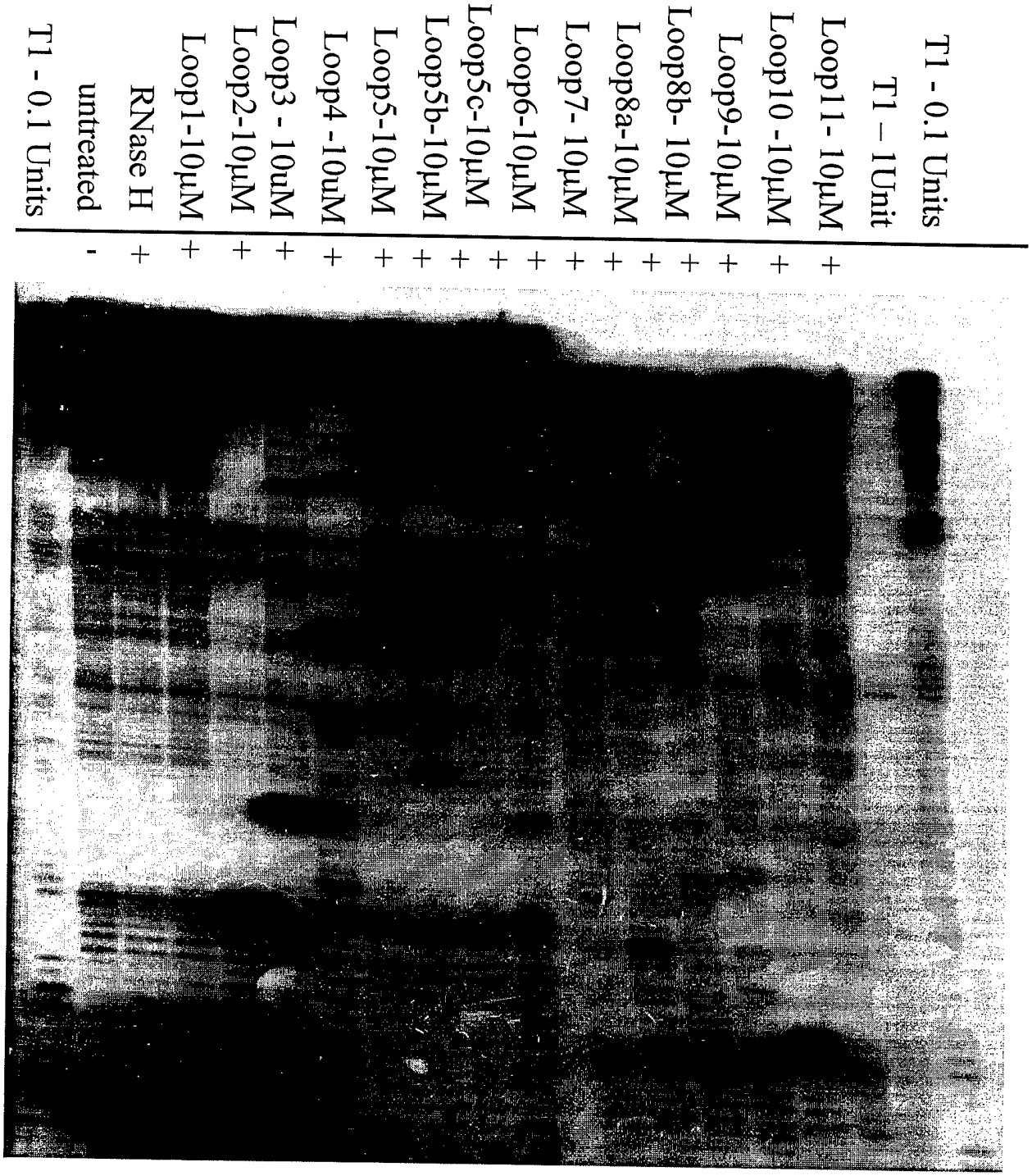


Figure 8

Random Hexamers correlate well to Confirmed Loop Regions in the 5'-UTR of the HER-2/neu mRNA

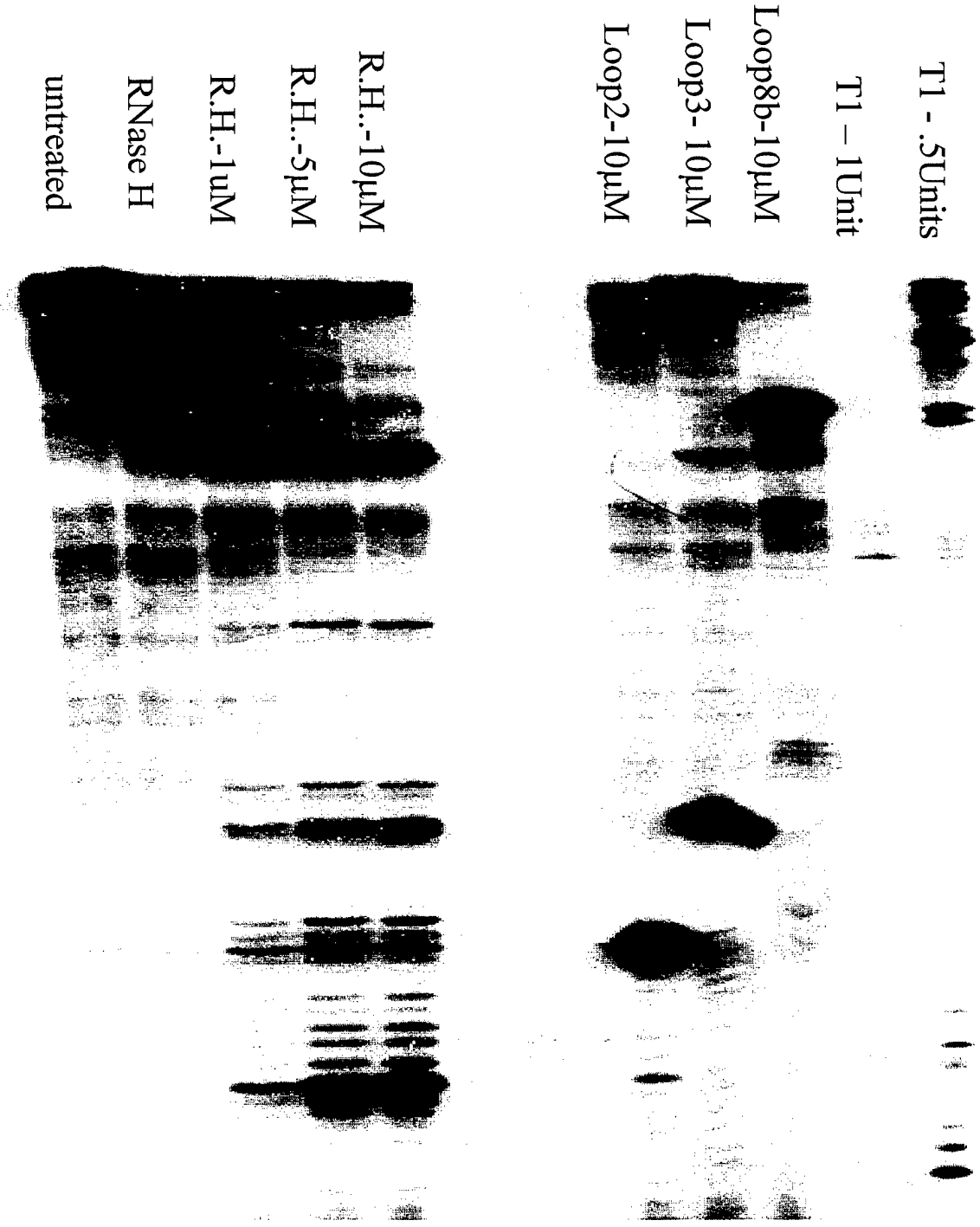
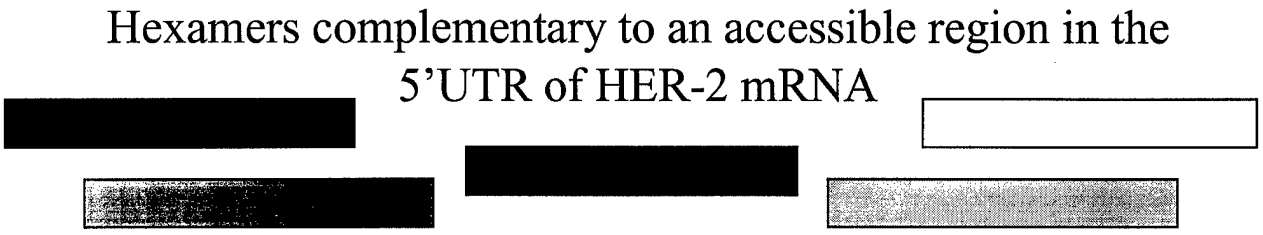
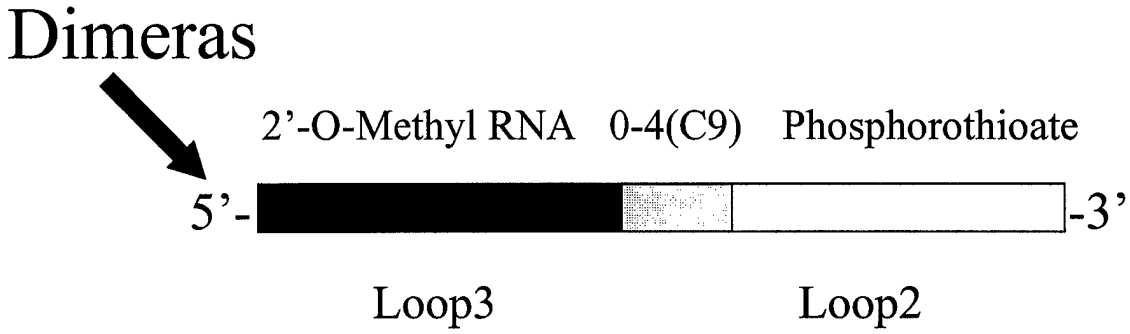


Figure 9

Design of Chimeric Antisense Molecules





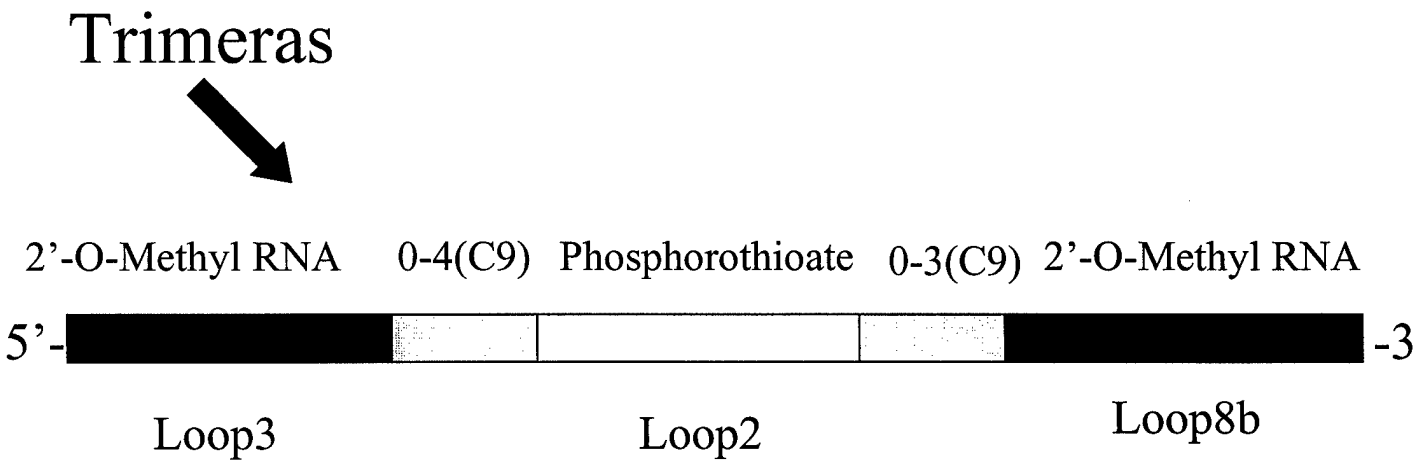


Figure 10

Enhanced Sensitivity of 5' region of mRNA to RNaseH by Antisense Dimera

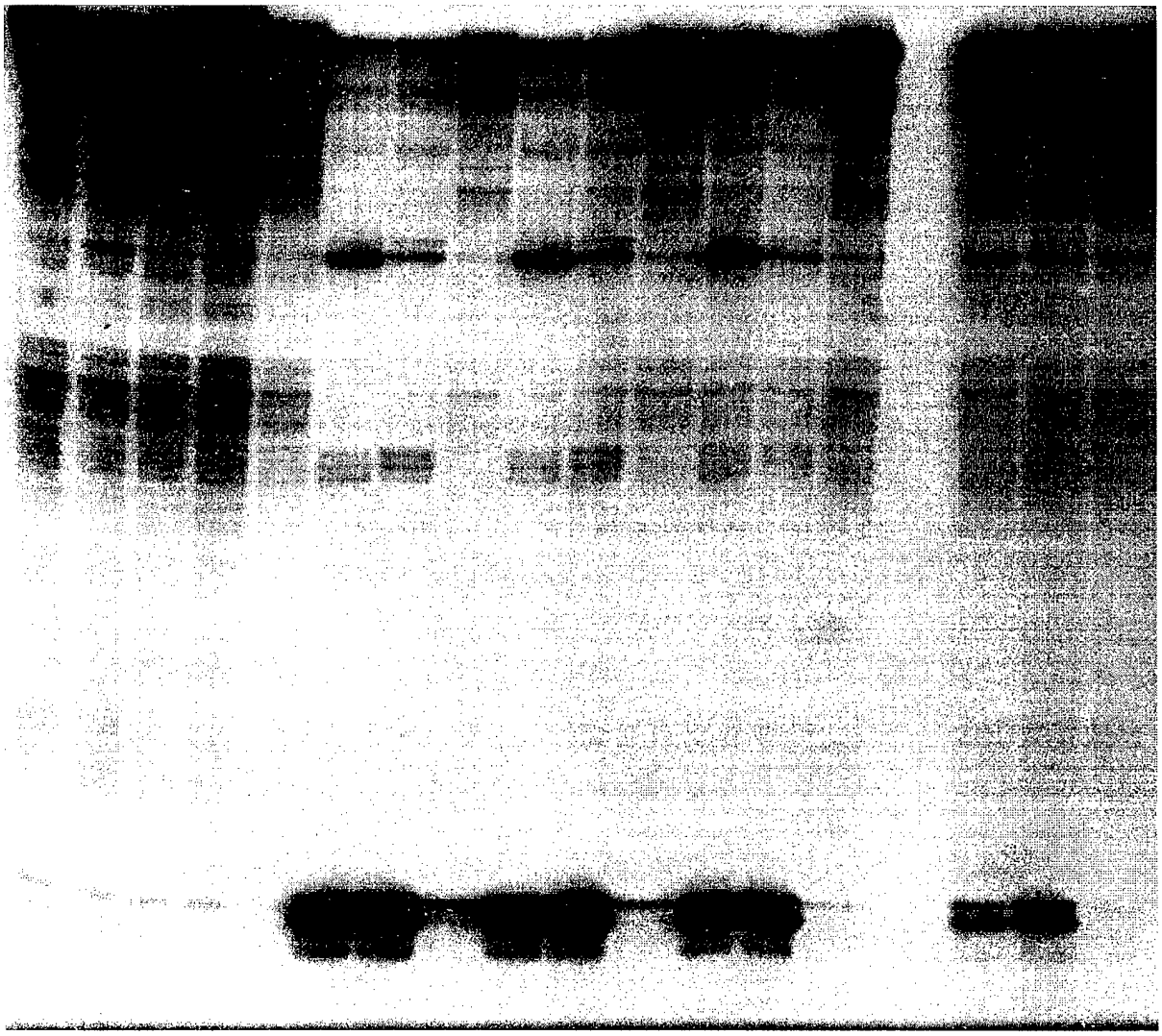


Figure 11

Dimeric Antisense molecules containing no spacers and 3 carbon 9 spacers most sensitize the 5' region of HER-2/neu mRNA to RNase H

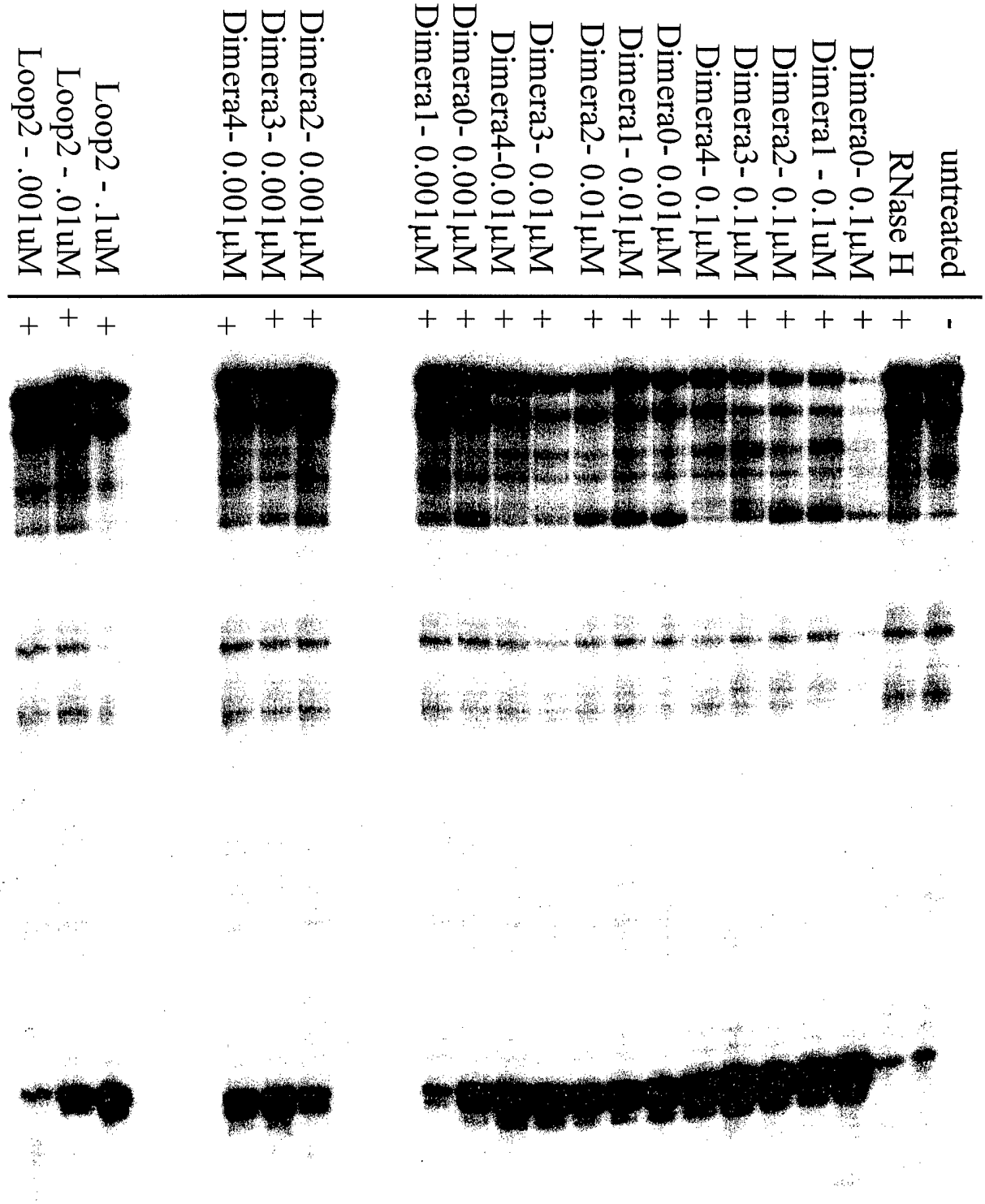


Figure 12

Phosphorothioate- Antisense oligonucleotides exhibit a weaker activity towards RNase H compared to DNA-Antisense oligonucleotides

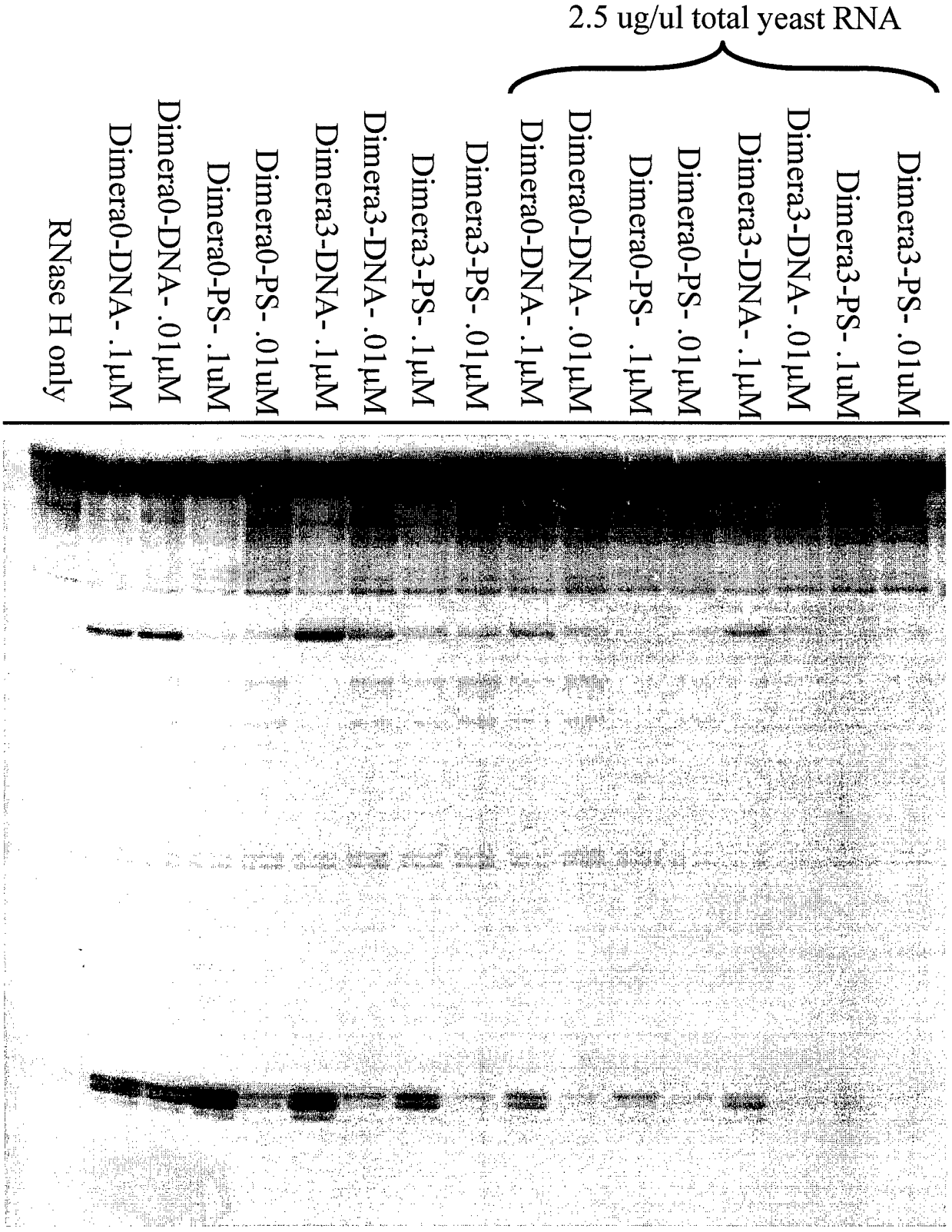


Figure 13

Dimera 0 and Dimera 3 are more specific than loop2 for the 5' UTR of the HER-2/neu mRNA in the presence of yeast total RNA

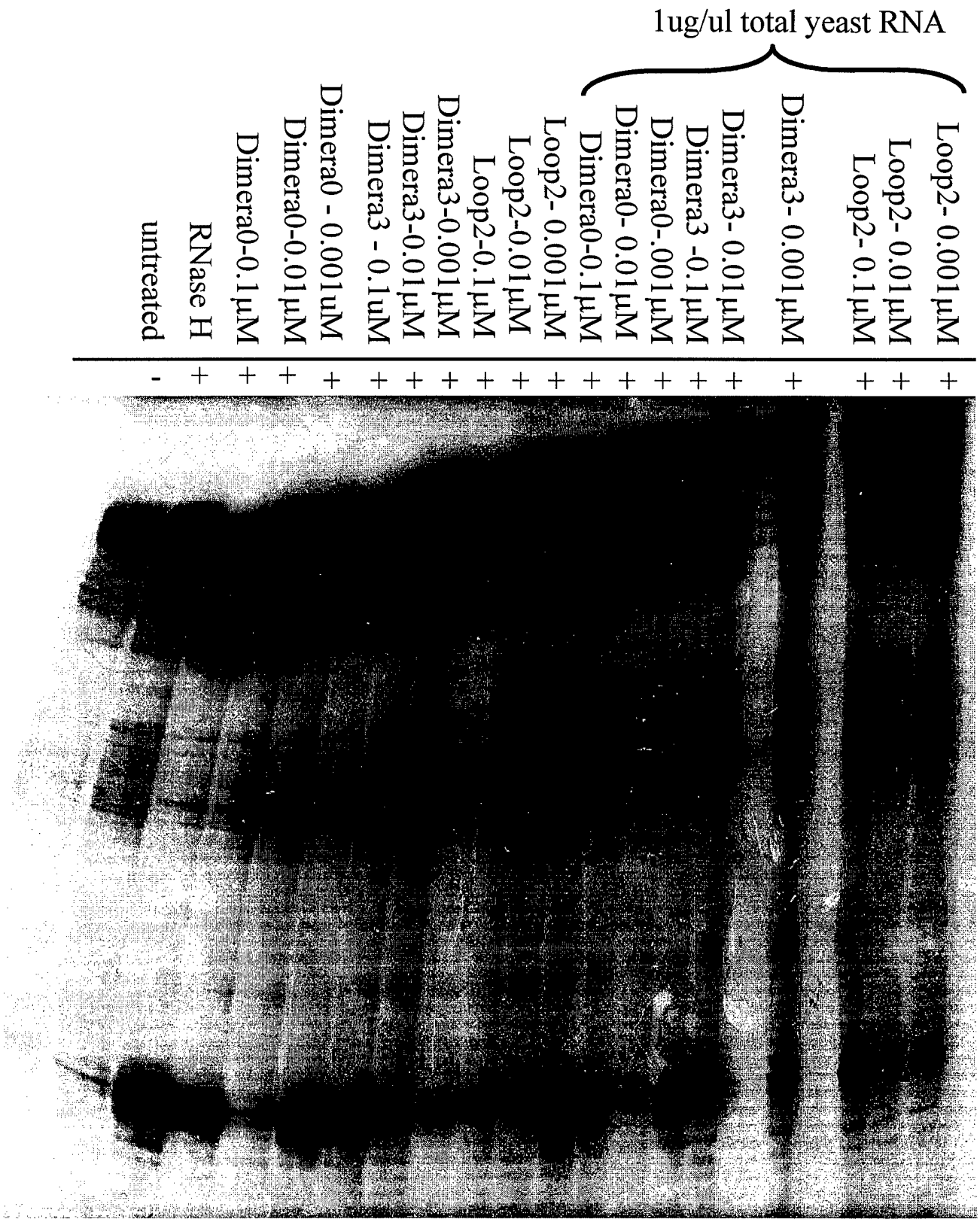


Figure 14

Trimeric antisense oligonucleotides do not enhance RNase H activity

Figure 15

Trimera0 does not exhibit additional specificity to the 5'-UTR of HER2/neu mRNA in the presense of total yeast RNA

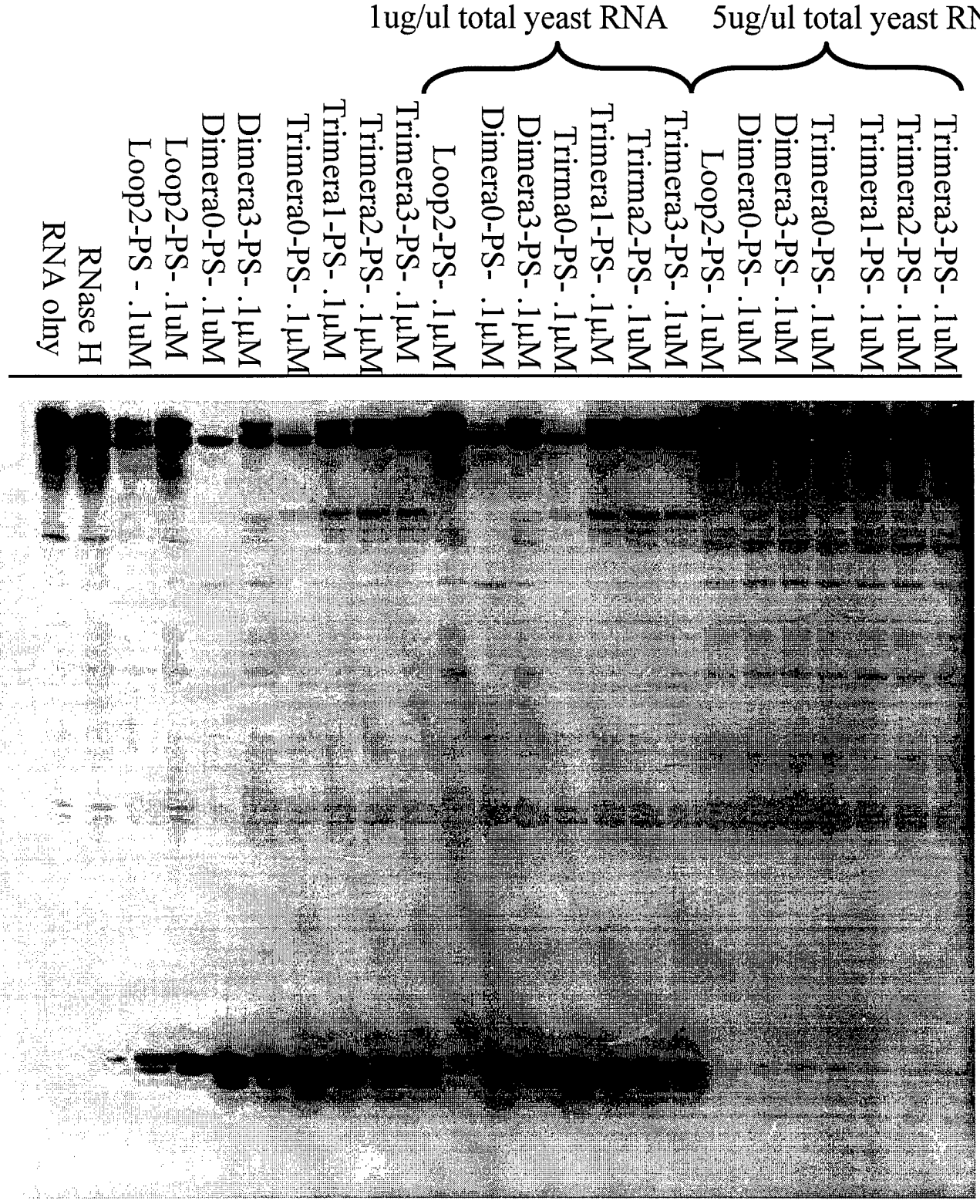


Figure 16

Construction of the T7-HER2 5'UTR Luciferase Vector

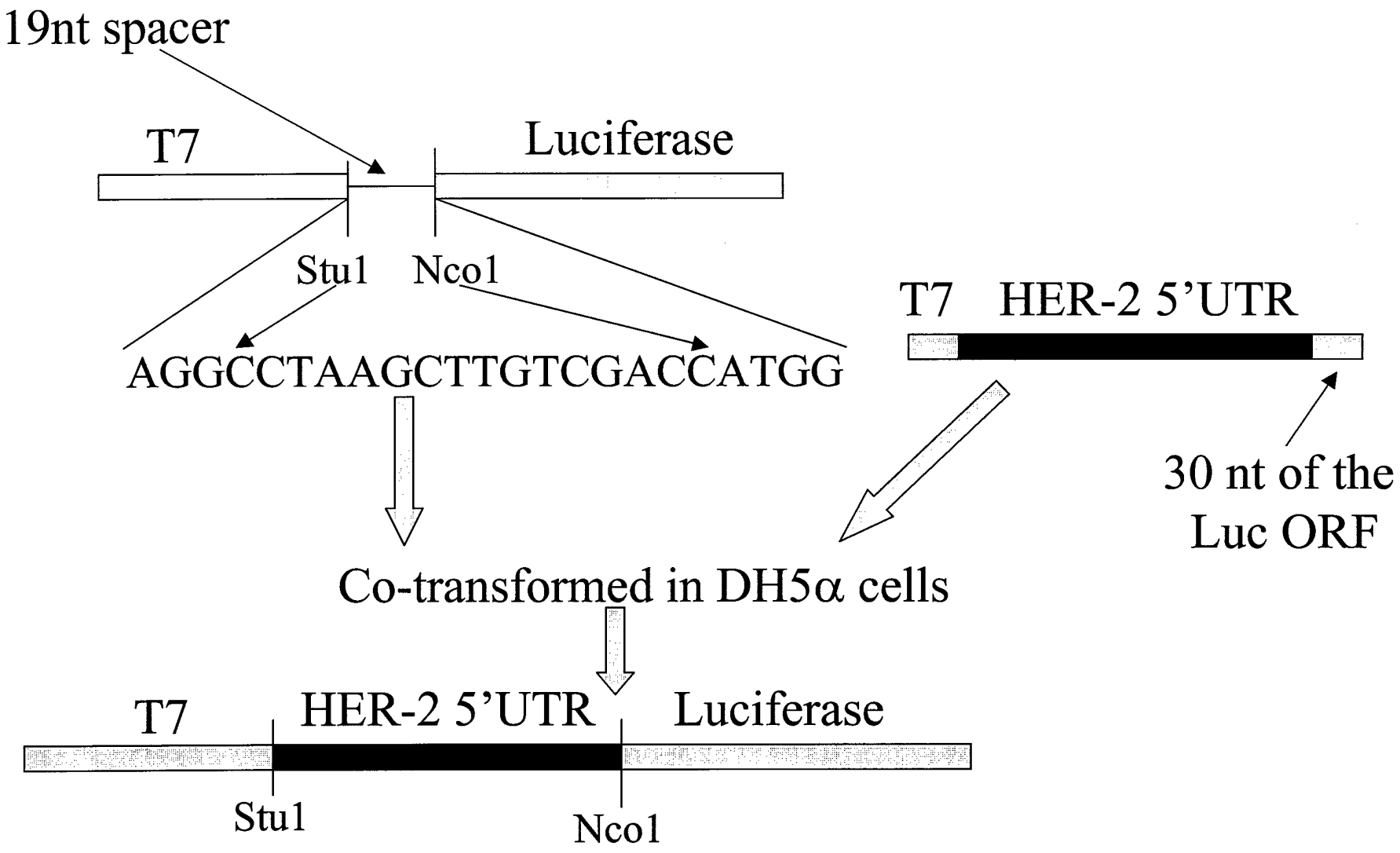


Figure 17

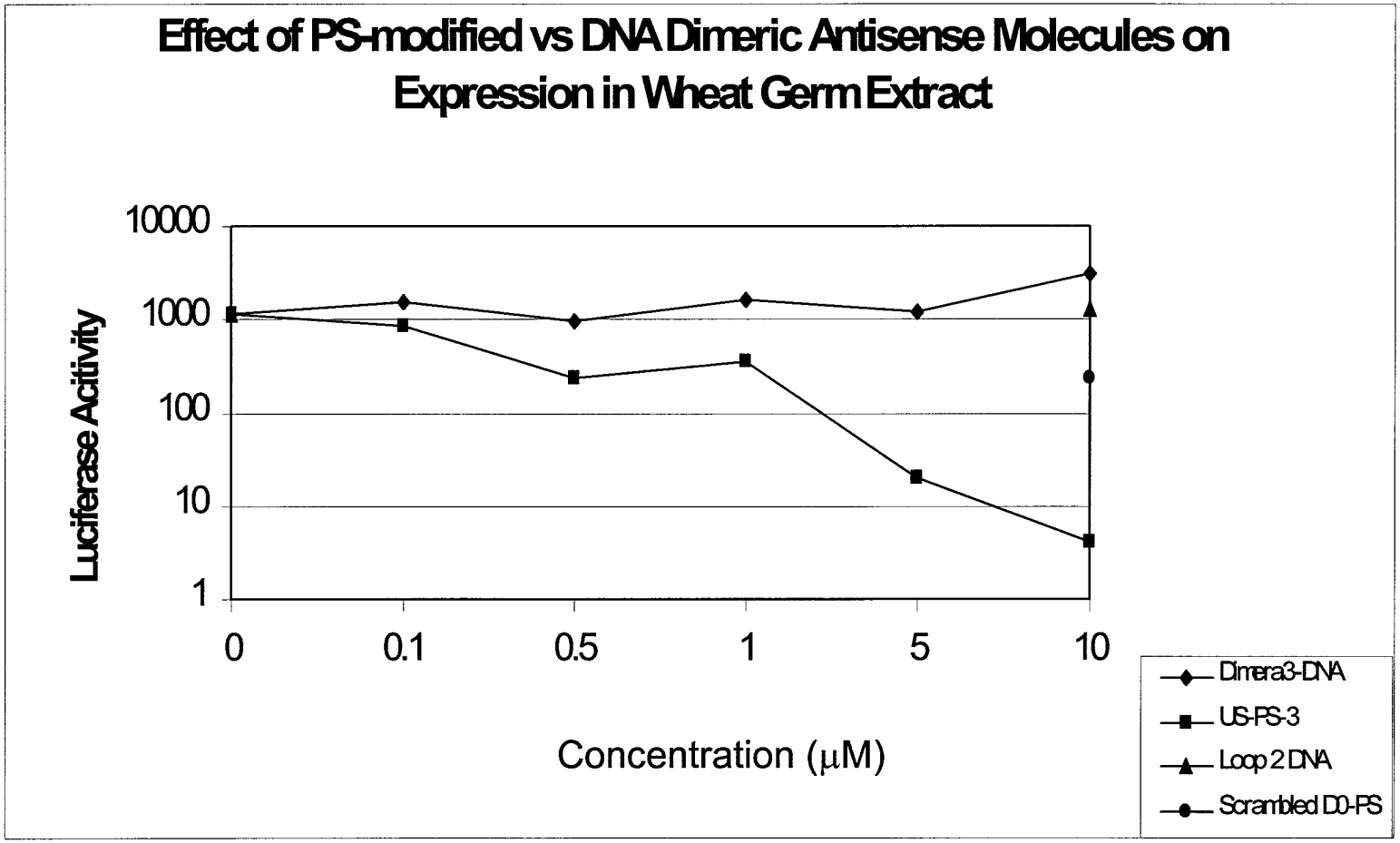

Effect of PS-modified vs DNA Dimeric Antisense Molecules on Expression in Wheat Germ Extract
Luciferase Acitivity
10000
1000
100
10
1
0
0.1
0.5
1
5
10
Concentration (μM)
Dimera3-DNA
US-PS-3
Loop 2 DNA
Scrambled D0-PS

Figure 18

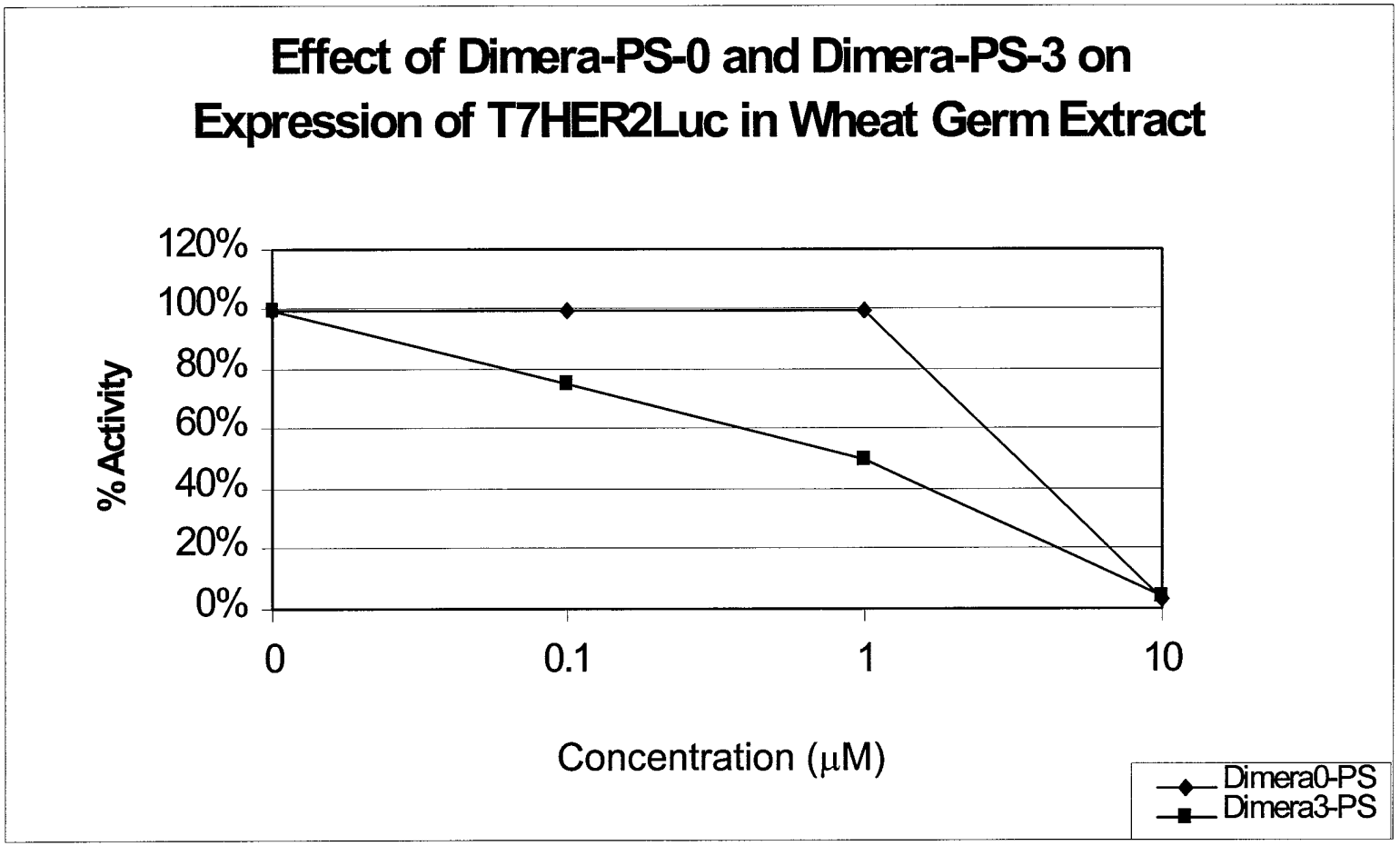

Effect of Dimera-PS-0 and Dimera-PS-3 on Expression of T7HER2Luc in Wheat Germ Extract
% Activity
120%
100%
80%
60%
40%
20%
0%
0
0.1
1
10
Concentration (μM)
Dimera0-PS
Dimera3-PS

Figure 19

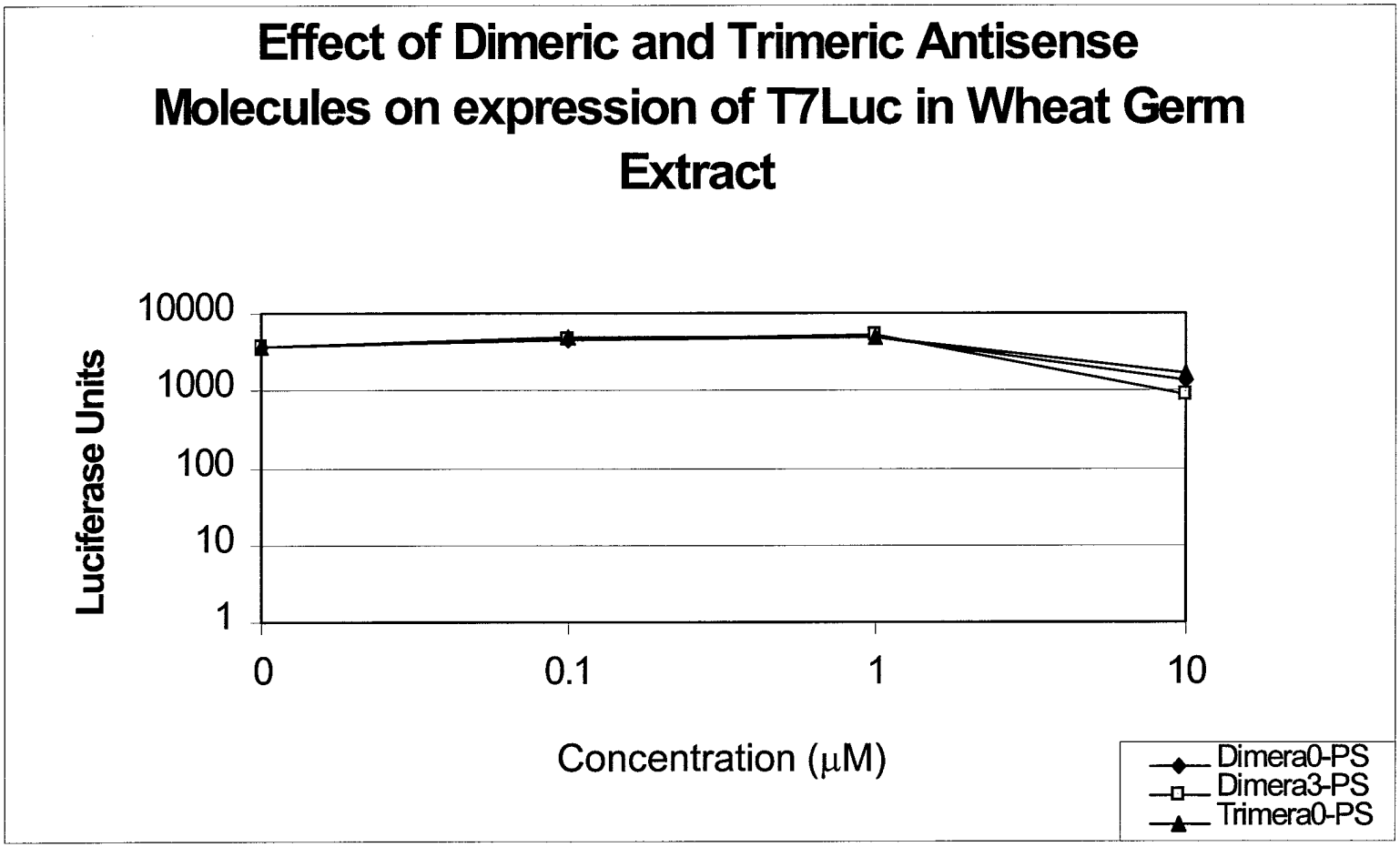

Effect of Dimeric and Trimeric Antisense Molecules on expression of T7Luc in Wheat Germ Extract
Luciferase Units
10000
1000
100
10
1
0
0.1
1
10
Concentration (μM)
Dimera0-PS
Dimera3-PS
Trimera0-PS

Figure 20

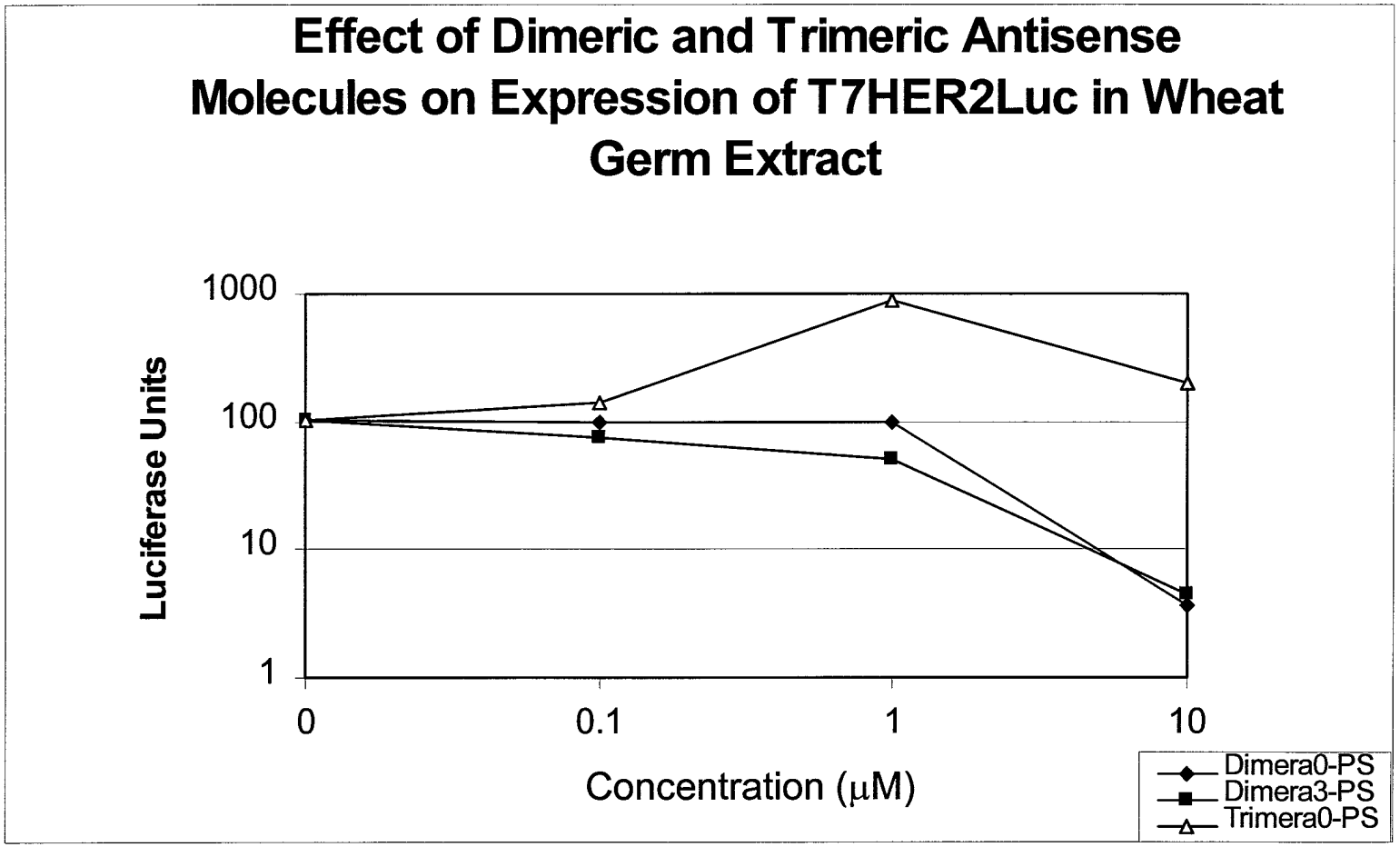

Effect of Dimeric and Trimeric Antisense Molecules on Expression of T7HER2Luc in Wheat Germ Extract
Luciferase Units
1000
100
10
1
0
0.1
1
10
Concentration (μM)
Dimera0-PS
Dimera3-PS
Trimera0-PS

Figure 21

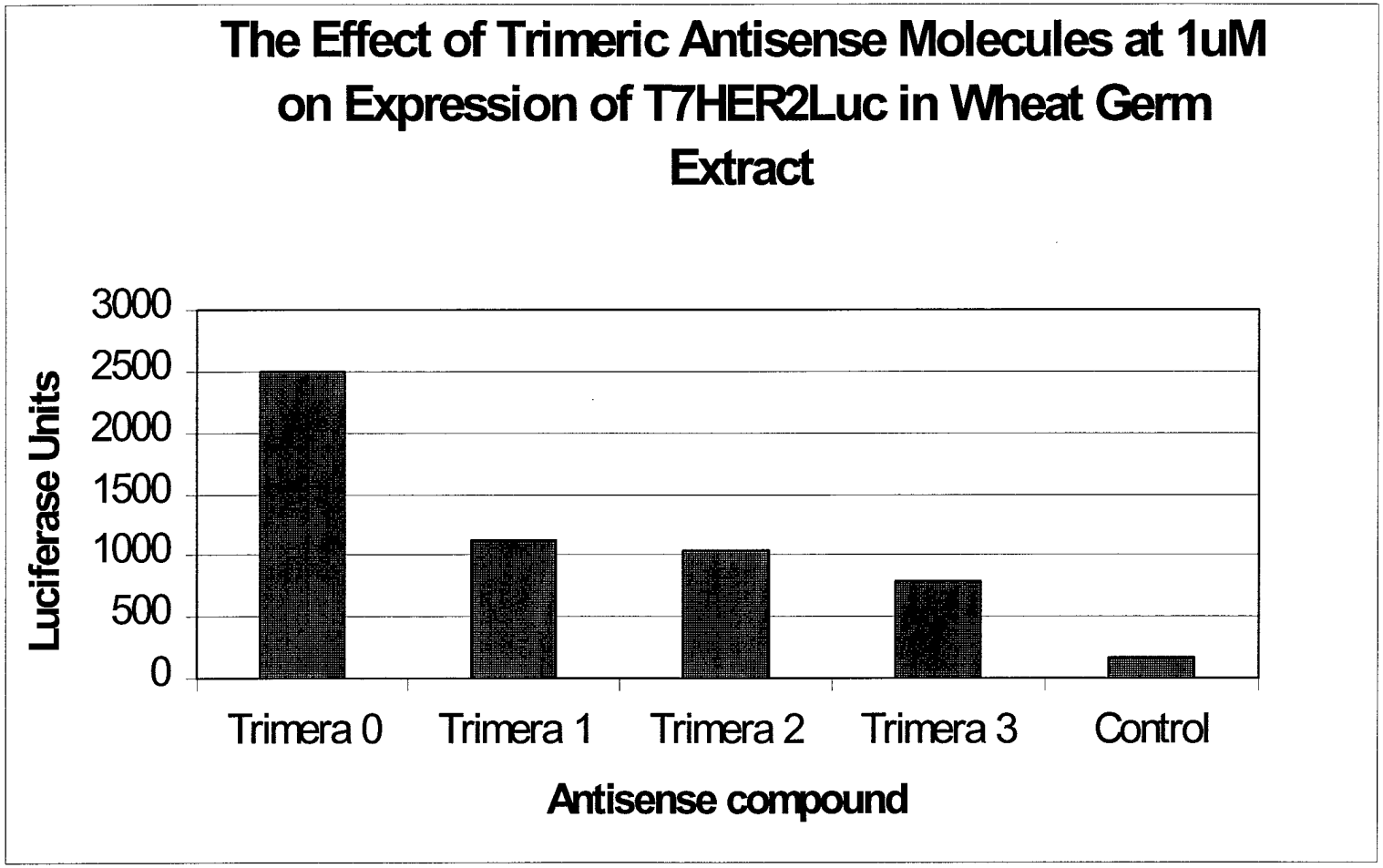

The Effect of Trimeric Antisense Molecules at 1uM on Expression of T7HER2Luc in Wheat Germ Extract
Luciferase Units
3000
2500
2000
1500
1000
500
0
Trimera 0
Trimera 1
Trimera 2
Trimera 3
Control
Antisense compound

Table 4

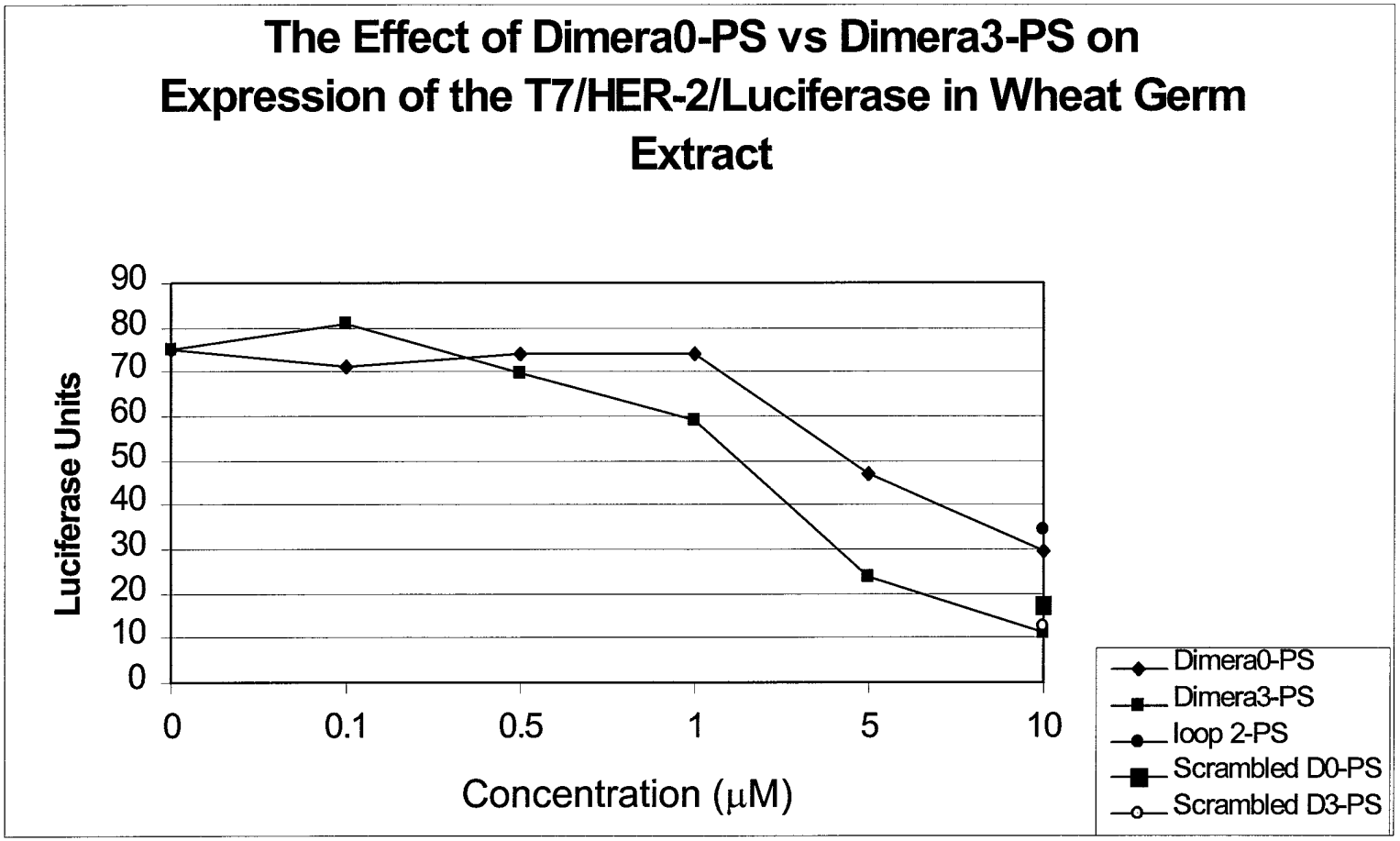

The Effect of Dimera0-PS vs Dimera3-PS on Expression of the T7/HER-2/Luciferase in Wheat Germ Extract
Luciferase Units
90
80
70
60
50
40
30
20
10
0
0
0.1
0.5
1
5
10
Concentration (μM)
Dimera0-PS
Dimera3-PS
loop 2-PS
Scrambled D0-PS
Scrambled D3-PS

Figure 22

[*In Press, Journal of Controlled Release*]

TARGETED PEG-BASED BIOCONJUGATES ENHANCE THE CELLULAR UPTAKE AND TRANSPORT OF AN HIV-1 TAT NONAPEPTIDE

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Running Title: PEG-based bioconjugates as intestinal carriers of peptides

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ABSTRACT

We previously described the enhanced cell uptake and transport of R.I-K(biotin)-Tat9, a large (~1500 Da) peptidic inhibitor of HIV-1 Tat protein, via SMVT, the intestinal biotin transporter. The aim of the present study was to investigate the feasibility of targeting biotinylated PEG-based conjugates to SMVT in order to enhance cell uptake and transport of Tat9. The 29kDa peptide-loaded bioconjugate (PEG:(R.I-Cys-K(biotin)-Tat9)$_8$) used in these studies contained 8 copies of RI-K(biotin)-Tat9 appended to PEG by means of a cysteine linkage. The absorptive transport of biotin-PEG-3400 (0.6-100 μM) and the bioconjugate (0.1-30 μM) was studied using Caco-2 cell monolayers. Inhibition of biotin-PEG-3400 by positive controls (biotin, biocytin, and desthiobiotin) was also determined. Uptake of these two compounds was also determined in CHO cells transfected with human SMVT (CHO/hSMVT) and control cells (CHO/pSPORT) over the concentration ranges of 0.05–12.5 μM and 0.003–30 μM, respectively. Nonbiotinylated forms of these two compounds, PEG-3350 and PEG:(R.I-Cys-K-Tat9)$_8$, were used in the control studies. Biotin-PEG-3400 transport was found to be concentration dependent and saturable in Caco-2 cells (K_m = 6.61 μM) and CHO/hSMVT cells (K_m = 1.26 μM). Transport/uptake was significantly inhibited by positive control substrates of SMVT. PEG:(R.I-Cys-K(biotin)Tat9)$_8$ also showed carrier-mediated transport kinetics in Caco-2 cells ((K_m = 6.13 μM) and CHO/hSMVT cells (K_m = 8.19 μM) cells. Maximal uptake in molar equivalents of R.I-Cys-K(biotin)Tat9 was 5.7 times greater using the conjugate versus the biotinylated peptide alone. Transport of the nonbiotinylated forms was significantly lower ($p < 0.001$) in all cases. The present results

demonstrate that biotin-PEG-3400 and PEG:(R.I-Cys-K(biotin)Tat9)$_8$ interact with human SMVT to enhance the cellular uptake and transport of these larger molecules and that targeted bioconjugates may have potential for enhancing the cellular uptake and transport of small peptide therapeutic agents.

KEYWORDS Tat, Caco-2, SMVT, peptide delivery, PEG conjugates, drug targeting

INTRODUCTION

The challenges associated with the effective oral delivery of novel and complex but poorly bioavailable therapeutic agents, including proteins, peptides, and oligonucleotides, stems primarily from their poor biopharmaceutical and physicochemical characteristics [1-5]. Using surfactant-based permeation enhancers that transiently modify the barrier properties of biological membranes is a common strategy for improving the intestinal permeability of poorly absorbed compounds. Despite initial enthusiasm, the invasive nature of this approach, lacks of precise control and the potential side effects have severely hampered their use [6,7]. Newer agents such as zonulin [8,9], that act by receptor-mediated, regio-specific and reversible mechanisms displaying considerably lower cytotoxicity and systemic side effects, now offer a promising tool in permeability enhancement. However, further studies are still necessary to fully establish their utility.

An alternative, non-invasive approach to facilitate intestinal drug absorption is to target specific absorptive transporter systems by chemical modification of drugs to prodrugs and analogues. For instance, our group previously demonstrated that

valacyclovir, the L-valyl ester prodrug of acyclovir, is a substrate of the intestinal proton-linked oligopeptide transporter, PepT1 [10,11]. Due to the low affinity, high capacity nature of PepT1, the interaction between valacyclovir and PepT1 results in a three to four-fold increase in the bioavailability of acyclovir. Despite accepting a wide range of endogenous and exogenous substrates with peptide-like structures, PepT1 facilitates only the apical transport of di- and tri-peptides, which makes it an unsuitable target for transporting larger peptides (> 5 amino acid residues) across the intestine [2,12].

Various vitamin and metal ion transporters have previously been used to facilitate transport of peptides. The cobalamin (vitamin B_{12}) receptor-mediated endocytotic system has been reported to be useful for the intestinal uptake of vitamin B_{12}-conugated luetinizing hormone releasing factor (LHRH)-analogs, erythropoietin, α-interferon and non-biodegradable polymeric nanoparticles (13,14). Despite its potential for oral peptides and protein delivery, this transport system suffers the drawback of limited uptake capacity (1 nmol per dose). The folate receptor-mediated endocytosis has been used in drug-targeting approaches for gene delivery (15), protein delivery (16), and targeting antisense oligonucletides (17) to various cell types. Additionally, the high affinity of folate conjugates for cell surface receptors ($K_D \sim 10^{-10}$ M) and the overexpression of folate receptors in cancer cells allows the selective delivery of folic acid-derivatized diagnostic and therapeutic agents to tumors. Transferrin receptors have been extensively studied for targeted drug delivery. Isolated from small intestinal mRNA and enterocytes, they primarily mediate the uptake of iron from the intestinal tract (up to 20 mg/day). In vitro and in vivo studies of transferrin-bound insulin showed receptor

mediated transport of the complex and significant reduction in plasma glucose levels of streptozotocin-induced diabetic mice (~ 28%) (18,19).

We recently reported the transport of R.I-K-Tat9 and its biotinylated analogue R.I-K(biotin)-Tat9, novel decapeptide inhibitors of the HIV-1 Tat protein across Caco-2 cell monolayers [20]. The absorptive transport of R.I-K-Tat9 was low (P_m ~1 x 10^{-6} cm/s) and not concentration dependent, whereas the permeability of R.I-K(biotin)-Tat9 was 3.2-fold higher (at 1 μM), active, concentration dependent and saturable (K_m = 3.27 μM). Using CHO cells transiently transfected with the human biotin transporter, hSMVT, we demonstrated the direct interactions between R.I-K(biotin)-Tat9 and SMVT (K_m = 1.0 μM, J_{max} = 227.2 pmol/mg protein/10 min). These interactions were inhibited in the presence of classical SMVT substrates, such as biotin, desthiobiotin, pantothenate and biocytin. Therefore, upon biotinylation, the transport properties of the passively transported R.I-K-Tat9 were substantially modified, rendering R.I-K(biotin)-Tat9 a substrate of SMVT. The results suggested that appending targeting moieties such as biotin might be a useful strategy for enhancing the intestinal permeability of large peptides. Since the K_m values of SMVT substrates are typically in the low micromolar range, the resultant saturation of the transporter may ultimately limit the dose of peptide that can be delivered. In order to overcome this potential limitation, we have investigated the possibility of using a poly(ethylene glycol) (PEG)- based bioconjugates to maximize the therapeutic payload of Tat-peptide transported [21]. The initial PEG-based bioconjugate was designed to (i) carry multiple copies of a drug attached by means of reducible disulfide bonds, (ii) have an extended half-life in blood or extracellular fluid. (iii) selectively release appended drug molecules inside the cell because of the differential

in reducing capacity between blood and inside cells and finally (iv) enhance cellular uptake of the drug. We recently demonstrated that a PEG bioconjugate containing multiple copies of a 10-amino acid Tat-peptide with an appended biotin molecule had an approximately 5-fold greater potency in preventing Tat protein-dependent gene expression in a cultured cell system compared to the nonconjugated biotin Tat-peptide [22]. This functional effect could be attributed to the non-specific amphiphilic properties of PEG and hydrophobicity of biotin, which possibly resulted in greater cell membrane adhesion/penetration of the conjugate [23,24]. The presence and functionality of SMVT was not evaluated in the experimental system. Definitive, mechanistic studies of the uptake of the biotinylated bioconjugate are lacking. In the present study, a mechanistic evaluation of the transport of biotin-PEG-3400 and PEG-(R.I-Cys-K(biotin)-Tat9)$_8$ was performed to assess the potential for using targeted bioconjugates as oral delivery vehicles.

MATERIALS AND METHODS

Materials

PEG:(R.I-Cys-K-Tat9)$_8$ and PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ were synthesized as described below. α, ω-Biotin-PEG-NHS (PEG M.W. = 3400) was purchased from Shearwater Polymers, Inc. (Huntsville, AL) and was used to prepare tritiated ([^{3}H]biotin-PEG-3400, specific radioactivity 1.542 mCi/mmol) as described below. [^{14}C]mannitol, [^{14}C]PEG-3350, and [^{3}H]acetic anhydride were obtained from NEN-Life Science Products (Boston, MA). The human sodium-dependent multivitamin transporter, hSMVT, subcloned in the mammalian expression vector, pSPORT, was kindly provided

by Dr. Puttur D. Prasad (Medical College of Georgia, Augusta, GA). All medium components and reagents for cell culture were obtained from Gibco Life Technologies, Inc. (Grand Island, NY). All other chemicals were from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

Synthesis of PEG:Tat9 Conjugate

R.I-Cys-K(biotin)-Tat9 was manually synthesized on PAL resin by Fmoc chemistry using reagents from PerSeptive Biosystems (Framingham, MA). For radiolabeling, the assembled peptide on the solid support (prior to N-acetylation) was allowed to react with tritiated acetic anhydride (specific activity 6.4 Ci/mmol) in the presence of coupling activation reagents BOP, HOBt, and DIEA. Acetylation of the peptide was driven to completion with an excess of unlabelled acetic anhydride. After cleavage from the solid support and ether precipitation, the radiolabeled peptide was purified by chromatography on Sephadex G-10 using phosphate-buffered saline (PBS) (0.15 M NaCl, 20 mM potassium phosphate buffer, pH 7.4) for elution.

The starting material for the carrier polymer was α, ω-diamino-PEG (NH_2-PEG-NH_2, MW 3400 Da) (Shearwater Polymers, Inc.). It was copolymerized with an equimolar amount of BOC-Asp using 1, 3-diisopropylcarbodiimide (DIPC) as the condensing reagent in the presence of 4-dimethylamino-pyridine (DMAP) and p-toluenesulfonic acid (pTSA) as catalysts. The resulting PEG-Asp copolymer was purified by precipitation with 10-volumes of cold ether. Using GPC/HPLC, the copolymer was estimated to have a molecular weight of ~29 kDa. The BOC protecting group on Asp was removed using trifluoroacetic acid (100%). The PEG-Asp conjugate

dissolved in PBS (pH 8.0) was reacted with 3-fold molar excess of the bifunctional coupling agent, SPDP (Pierce Chemical Co., Rockford, IL), dissolved in dimethylsulfoxide, for 6 hours. This coupling reagent essentially converts an amino group to a thiol group protected by the thiopyridine (TP) group. The conjugate was precipitated with ether again to remove excess SPDP reagents. The concentration of SPDP-reacted polymer was determined on an aliquot, based on the release of 2-thiopyridine (TP) using DTT. In this analytical procedure, an aliquot of the reaction mixture was reacted with excess DTT (pH 8.0) for 15 minutes and the amount of TP liberated was quantified spectrophotometrically at 343 nm [26]. The SPDP-treated polymer was reacted with excess R.I-Cys-K(biotin)-Tat9 in PBS (pH 7.5) for 16 hours and coupling of peptide to the carrier was monitored at 343 nm. The PEG:R.I-Cys-K(biotin)-Tat9 conjugate was maintained under argon at –20°C until use for transport experiments.

Structure of the Bioconjugate Used in the Present Study

The PEG carrier used in the present study consisted of repeating units of PEG-3400-Asp appended to R.I-Cys-K(biotin)-Tat9, tritiated at the N-terminus, via a bioreversible disulfide bond. The structure of the bioconjugate used in these studies is shown in Figure 1.

Biotin-PEG-3400 Tritiation

The succinimide ester of biotin-PEG-3400 (biotin-PEG-CO_2-NHS, 15.6 μmol) (Shearwater Polymers, Inc.) dissolved in 4 ml sodium carbonate (50 mM, pH 9.0) was

added to diamino-butane (1.64 mmol) and the mixture stirred overnight. Following aqueous dilution (30 ml), the resultant product was extracted three times with methylene chloride (30 ml) and reduced to 0.5 ml using a rotary evaporator. Subsequently, the product was precipitated with ethyl ether, purified using HPLC with a SEC column and dried under speed vacuum. It was then dissolved in 0.5 ml DMF and reacted with [^{3}H]acetic anhydride using BOP/HOBt as coupling reagents. The product was ether precipitated and dried under air.

Cell Culture Protocols

The Caco-2 cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD) at passage 25. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, penicillin (2%), and streptomycin (2%), in an atmosphere of 95% air and 5% CO_2 at 37 °C. Culture medium was changed every other day and cells were passed every 3-5 days by trypsinizing cells with 0.05% trypsin and 0.53 mM EDTA at 37°C for 10 minutes. Confluency was determined using a Zeiss Telaval 31 inverse phase contrast microscope. After harvesting at 90% confluency, cells were seeded at a density of 63,000/cm^2 on Snapwell inserts with 0.4 μm pore diameter (Corning-Costar, Cambridge, MA). Cells at passage number 27-30 cultured for 3-4 weeks after seeding were used for all experiments. For cell density determinations, the cell suspensions grown in various T-flasks were combined into a centrifuge tube and shaken gently for even distribution. Using aseptic technique, the cell suspension were stained with trypan blue and counted using a hemocytometer. Appropriate dilution was made with fresh

media to bring cell density to 1.25 x 10^5 cells/ml (target cell density on Snapwell: 63,000/cm^2). CHO cells were obtained from the ATCC at passage 4 and cultured under conditions similar to Caco-2 cells, except that trypsinization during cell passage was done for 2 minutes instead of 10 minutes. Upon reaching confluency, the cells were transfected with the human sodium-dependent multivitamin transporter, hSMVT, subcloned in the mammalian expression vector, pSPORT. The cells were transfected with hSMVT or pSPORT (vector control) by Lipofectamine according to the manufacturer's instructions (GIBCO BRL). Briefly, after harvesting at 90% confluency, the cells were seeded at a density of 3 x 10^5 cells/well in 12-well culture plates and incubated at 37°C for 24 hours. For each well, 1 μg of DNA was mixed gently with 200 μl of serum-free medium and 10 μg of Lipofectamine reagent. The mixture was incubated at room temperature for 15 minutes and transferred to each well. Then 0.8 ml of serum-free medium was added to the mixture. After a 5-hour incubation at 37°C, the transfection mixture was removed and replaced with 1 ml of complete growth containing 10% FBS.

Caco-2 Cell Validation Studies

In order to validate the barrier properties of the Caco-2 cell monolayers, the cells were rinsed twice with pre-warmed Ringer's buffer with glucose buffer (pH 7.4, iso-osmotic) and trans-epithelial electrical resistance (TEER) was measured using an EVOM epithelial voltmeter equipped with an Endohm electrode (World Precision Instruments, Sarasota, FL) both before and after experimentation. In addition, marker compounds were selected to characterize the monolayers. Specifically, mannitol, an indicator of

paracellular transport, and poly(ethylene glycol)-4000, a classical nonabsorbable marker, were used as indicators of the barrier properties of the monolayers. Tritium exchange between the ^{3}H-labeled compounds and H_2O was quantified by air-drying 100 µl aliquots of solutions containing the radiolabeled compound at their respective experimental concentrations in a fume hood overnight. The remaining radioactivity was compared to controls before evaporation.

Caco-2 Cell Transport Studies

An Ussing-type diffusion chamber system (Harvard Apparatus, Natick, MA) was used to perform transport studies. The apical solution was a MES Ringer's buffer (pH 6.5) consisting of 114.0 mM NaCl, 5.0 mM KCl, 1.1 mM $MgSO_4$, 1.25 mM $CaSO_4$, and 15 mM 2-(N-morpholino)-ethanesulfonic acid (MES). The basolateral solution was a Ringer's buffer (pH 7.4) consisting of 114.0 mM NaCl, 5.0 mM KCl, 1.65 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.1 mM $MgSO_4$, and 1.25 mM $CaSO_4$. Both buffers contained 25 mM glucose and were adjusted to 290 mOsm/kg using a vapor pressure osmometer (Wescor Inc, Logan, UT). For transport experiments using filters with cell monolayers, the inserts were rinsed with Ringer's buffer containing 25 mM glucose three times over a 20 min period at room temperature. The TEER was measured for each monolayer before it was mounted onto the diffusion chamber, which was pre-warmed to 37 °C by placement on the heating blocks. The volume of each half-chamber was 5 ml and the surface area of the Snapwell™ filters available for drug transport was 1 cm^2. The fluids in the chambers were circulated using a gas lift mechanism with 5% CO_2/95% O_2; flow rate was adjusted to 10 ml/min and monitored using a J&W

ADM2000 gas flow meter (Fisher Scientific). The donor solution consisted of radiolabeled permeant in the apical buffer [apical (AP) to basolateral (BL) study]. Starting at 30 min, samples were taken from the receptor chamber every 15 min until the end of the experiment (105 min). A minimum of three monolayers was used to determine the permeability of test compound in each study and sample analysis was performed using scintillation counting (Perkin Elmer, Gaithersburg, MD).

CHO Cell Uptake Studies

CHO cells transiently transfected with hSMVT (CHO/hSMVT) were washed twice with 25 mM uptake buffer, containing 25 mM Hepes/Tris pH 7.5, 140 mM NaCl, 5.4 mM KCl, 1.8 $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose. Subsequently, the cells were incubated with the permeant at 37°C for 10 minutes. Washing the cells three times with ice-cold buffer stopped uptake. Non-specific uptake was measured in parallel experiments with the control pSPORT vector-transfected CHO cells. Finally, the cells were solubilized by 0.1% v/v Triton X-100, and 0.6 ml was used for scintillation counting. From the remaining volume, 10 µl of solution was taken from each well and protein concentration was determined using the Bio-Rad reagent according to the manufacturer's instructions. Bovine serum albumin was used as the standard.

Concentration Dependence of biotin-PEG-3400

The concentration dependence of biotin-PEG-3400 (0.6-100 µM) permeability was determined by evaluating its AP to BL or absorptive transport across Caco-2 cell monolayers. PEG-3350 (0.1 – 100 µM) was used in the control experiments. The uptake

of biotin-PEG-3400 (0.05–12.5 μM) was also studied in CHO/hSMVT cells with simultaneous determination in control CHO/pSPORT cells. PEG-3350 (0.05-10 μM) uptake in CHO/hSMVT cells was determined in the control studies.

Inhibition of biotin-PEG-3400

The involvement of SMVT in the Caco-2 cell monolayer transport of biotin-PEG-3400 was investigated through inhibition studies that were performed by co-incubating biotin-PEG-3400 with competitive substrates of SMVT in the donor solution (AP) of the diffusion chambers. [^{3}H]-biotin-PEG-3400 was co-incubated with 10 μM and 20 μM concentrations of biotin, biocytin and desthiobiotin and a putative transport competitor, biotin-PEG-biotin.

Concentration Dependence of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$

Following the permeability determinations of biotin-PEG-3400, the absorptive transport of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ (0.1-30 μM) was evaluated across Caco-2 cell monolayers and permeability values determined. Nonbiotinylated PEG:(R.I-Cys-K-Tat9)$_8$ (0.1-30 μM) was used in the control studies. The uptake of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ (0.003-30 μM) was also studied in CHO/hSMVT cells. PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ uptake in CHO/pSPORT cells and uptake of PEG:(R.I-Cys-K-Tat9)$_8$ (0.04-45 μM) in CHO/hSMVT were estimated in the control experiments. For all the conjugate transport studies, permeability measurements were made as a function of peptide concentration.

Inhibition of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ Conjugate

The uptake of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ conjugate (0.1 μM) was studied in CHO/hSMVT cells in the presence of biotin, pantothenic acid, desthiobiotin, biocytin, biotin-PEG-3400 and biotin-PEG-biotin (50 μM) and compared to the control, the conjugate without inhibitors.

Interpretation of Permeant Concentrations

The transport experiments were performed using a PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ conjugate with a specific activity equal to 12.9 Ci/mol of R.I-Cys-K(biotin)-Tat9 or 0.10 Ci/mmol of the conjugate. Standard and donor solutions for the transport studies were prepared based on the concentration of R.I-Cys-K(biotin)-Tat9. Since all calculations were performed based on the peptide concentration, the results from Caco-2 and CHO cell experiments represent the transport of PEG-conjugated or free peptide, R.I-Cys-K(biotin)-Tat9, and not moles of conjugate. The Michaelis-Menten kinetic parameters for Caco-2 transport and CHO uptake were used as estimated.

Stability of the Conjugate in the Experimental Buffer With and Without Cells Present.

The stability of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ in the experimental buffer with and without cells was determined in the following manner. One half milliliter of a 15 μM PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ solution prepared in the experimental buffer was incubated for 2 hours at 37°C. A control study without PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ was run in parallel. One half-milliliter samples were also withdrawn from the apical and

basolateral chambers following the last sample taken at 105 minutes for studies with initial donor concentrations equal to 0.1 μM and 25 μM. A 100-μl aliquot of the solution was then placed on a Microcon™ filter (molecular weight cut-off, MWCO = 3000 Da) (Amicon Inc., Beverly, MA) and centrifuged at 12,000 g for 30 minutes. For the buffer stability study, the residual radioactivity in the filtrate and in the control buffer was less than 5 CPM (counts/minute), indicating that all of the tritiated peptide (M.W. = 1500 Da) was attached to the conjugate. Similarly, the conjugate was stable in the apical chamber with 98.8% of the peptide attached to the polymer at the end of the experiment. On the other hand, 49.5 % and 51.2 % of R.I-Cys-K(biotin)Tat9 was cleaved from PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ at 0.1 μM and 25 μM (n = 3), respectively, when measured in the basolateral chamber.

Data Analysis

The effective permeability, P_e, (cm/sec) was determined using the equation

$$P_e = dC/dt * V_r/AC_0$$

where V_r is the volume of the receptor chamber (5 ml), A is the surface area of the filter (1 cm^2), C_0 is the initial drug concentration, and dC/dt is the flux (J) determined by the linear slope of receptor drug concentration versus time plot after correcting for dilution. The Michaelis-Menten kinetic parameters for the Caco-2 cell monolayer transport studies were estimated by performing weighted non-linear regression with Scientist software (MicroMath, Salt Lake City, Utah) using the following equation:

$$P_e = \frac{P_c}{1 + \frac{C}{K_m}} + P_m$$

where P_e is the effective permeability of the substrate transported, C is the substrate concentration and P_m and P_c represent the passive and carrier-mediated component of the permeability. The data from inhibition studies were transformed into Lineweaver-Burke plots to determine if the inhibition behavior was competitive or non-competitive. K_i values were determined using the equation

$$P_e = \frac{P_c}{C + K_m(1 + \frac{I}{K_i})} + P_m$$

where P_e, the effective permeability (cm/s), is related to the maximum flux, J_{max}, drug concentration, C, concentration of the inhibitor, I, affinity constant, K_m, and the estimated inhibition constant, K_i. Data used for the above plots were weighted using $1/SEM^2$. The kinetic parameters for CHO cell studies were estimated by performing non-linear regression using the equation $J = J_{max} * [C]/(K_m + [C])$, where J is the rate of permeant uptake, J_{max} is the maximal uptake velocity and [C] is the substrate concentration. Data were weighted using $1/S.E.M^2$. Statistical analyses were performed using Jandel SigmaStat, version 2.03. One-way analysis of variance was used to test the difference in the mean values of uptake using $p < 0.05$ as the significance level for all tests.

RESULTS

Caco-2 Cell Monolayer Validation Studies

The Caco-2 cell validation studies were performed to determine the functional integrity of the monolayers. TEER measurements indicated values of 300-350 $\Omega*cm^2$, both before and after the transport experiments. The permeability of mannitol (5 μM) was determined to be 1.42 (± 0.28) x 10^{-6} cm/sec and that for PEG-3350 was ~1 x 10^{-6} cm/sec. These results indicate that the cell monolayers were not leaky or damaged. The studies performed to measure tritium exchange indicated no significant differences in the radioactivity of the air-dried samples when compared to the controls, indicating the absence of proton transfer. For all the compounds studied, concentrations in the receptor chamber were linear with time for the duration of the experiments.

Biotin-PEG-3400 Transport Studies

Concentration Dependence of Biotin-PEG-3400

The absorptive transport of PEG-3350 across Caco-2 cell monolayers was low (~1 x 10^{-6} cm/sec), not concentration dependent and the probable result of passive diffusion. However, biotin-PEG-3400 transport across Caco-2 cells followed concentration dependent and saturable kinetics (Figure 2). The Michaelis-Menten kinetic parameters for biotin-PEG-3400 transport are presented in Table 1. The low K_m (6.61 μM) value for biotin-PEG-3400 transport indicated the potential involvement of a high affinity, low capacity transporter system. The uptake of biotin-PEG-3400 in CHO/hSMVT cells was concentration dependent and saturable (Figure 3) with estimated K_m and J_{max} values of 1.26 ± 1.01 μM and 23.46 ± 5.04 pmol/mg protein/10 min,

respectively. Transformation of the uptake data into an Eadie-Hofstee plot ($r = 0.9047$) (Figure 3 inset) indicated that the kinetics of biotin-PEG-3400 matched a single, saturable carrier model. Biotin-PEG-3400 uptake was significantly lower ($p < 0.01$) in the control CHO/pSPORT cells (4.22 pmol/mg protein/10 min) compared to CHO/hSMVT cells, but higher than PEG-3350 uptake in CHO/hSMVT cells (< 1 pmol/mg protein/10 min) (Figure 3).

Inhibition of biotin-PEG-3400

Biotin-PEG-3400 inhibition studies across Caco-2 cell monolayers were performed with the known SMVT substrate biotin, its structural analogues desthiobiotin and biocytin, and putative competitor, biotin-PEG-biotin. The Lineweaver-Burke plots suggested competitive inhibition of biotin-PEG-3400 transport by biotin (Figure 4a), desthiobiotin (Figure 4b), biocytin (Figure 4c), and biotin-PEG-biotin (Figure 4d) with inhibition constant (K_i) values of 6.78 μM, 11.47 μM, 14.01 μM, and 19.08 μM, respectively.

PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ Conjugate Transport Studies

Concentration Dependence of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$

The absorptive permeability of the PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ conjugate through Caco-2 cells was concentration dependent and saturable (Figure 2). The Michaelis-Menten kinetic parameters for PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ transport are presented in Table 1. In the control experiments, transport of PEG:(R.I-Cys-K-Tat9)$_8$ conjugate was low (< 10^{-7} cm/sec). PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ uptake in CHO/hSMVT cells exhibited concentration dependent and saturable kinetics with

estimated K_m and J_{max} values of 8.19 μM and 1288.18 pmol/mg protein/10 min, respectively (Figure 3). In the control studies, uptake of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ in CHO/pSPORT cells and PEG:R.I-Cys-K-Tat9 uptake in CHO/hSMVT cells were low (< 1 pmol/mg protein/10 min).

Inhibition of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ Conjugate

Uptake of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ in CHO/hSMVT cells was significantly lower ($p < 0.05$) compared to the control in the presence of known SMVT substrates biotin, pantothenic acid, desthiobiotin, biocytin and compounds evaluated in this study, biotin-PEG-3400 and biotin-PEG-biotin (Figure 6).

Discussion

Previous results from our laboratory [10,11] have illustrated the utility of designing peptide prodrugs that target specific intestinal transporters, such as PepT1, in order to enhance their bioavailability and therapeutic significance. Typically, substrates of intestinal transporters are smaller than ~600 Da. Recently, we demonstrated that the permeability of a substantially larger peptide, the anti-HIV Tat peptide, R.I-K(biotin)-Tat9, was significantly enhanced by conjugating a biotin to the peptide [20]. Even though its passive uptake and transport was significantly enhanced, the addition of a biotin also made the peptide a substrate for a carrier-mediated pathway whose transport was facilitated by SMVT. The affinity of the biotinylated peptide for SMVT was found to be low, potentially limiting the amount of peptide that could be delivered. In order to augment the total transport of the peptide, a PEG-peptide conjugated (PEG:(R.I-Cys-

K(biotin)-Tat9)$_8$, containing ~8 appended units of the peptide was synthesized and its transport mechanism was investigated.

SMVT is expressed in the placenta, intestine, heart, brain, lung, liver, kidney and pancreas and has been cloned from rats, rabbits, and humans [28-32]. Three distinct variants (II, III, and IV) of the placental SMVT (I) have been identified in rat small intestine [32]. However, unlike these variants, the Caco-2 SMVT cDNA, codes for an electrogenic protein of 635 amino acids with 12 transmembrane domains that is identical to the SMVT expressed in the human placental choriocarcinoma cell line (JAR) [29]. Using functional and molecular assay techniques, our previous results confirmed the expression of SMVT in Caco-2 and transiently transfected CHO/hSMVT cell systems [20].

Since our goal is to transport a substantially large conjugate (~29 kDa) across the intestine, initial feasibility studies using a smaller PEG (~3400 Da) with an appended biotin molecule (biotin-PEG-3400) were initiated. PEG-3350, which is typically used in many transport studies as a non-absorbable marker, served as the control. The absorptive permeability of biotin-PEG-3400 across Caco-2 cell monolayers was found to be concentration dependent, saturable, and inhibited by substrates of SMVT suggesting the involvement of SMVT whereas, in the control studies, PEG-3350 exhibited minimal transport consistent with previous reports [33-35]. The low K_m (6.61) of biotin-PEG-3400 was similar to the values reported for the intestinal transport of biotin (~5-11 μM) [20,31-32,36], pantothenate (~5 μM) [29], and R.I-K(biotin)-Tat9 (~3 μM) [20]. In transfected CHO cells, the uptake of biotin-PEG-3400 was concentration dependent, saturable and significantly greater in CHOhSMVT cells ($p < 0.01$) than CHO/pSPORT

cells (Figure 3). The K_m value (1.26 μM) from the uptake study was consistent with the previously reported values in SMVT-overexpressed cell systems for other SMVT substrates, such as biotin (1.3 μM) [20], R.I-K(biotin)-Tat9 (1.0 μM) [20], and pantothenate (1.6 μM) [29,30]. The uptake of passively transported PEG-3350 was significantly lower ($p < 0.001$) in CHO/hSMVT and CHO/pSPORT cells (< 1 pmol/mg protein). The Caco-2 cell monolayer transport of biotin-PEG-3400 was competitively inhibited by SMVT substrate biotin, and its analogues, desthiobiotin, and biocytin. The addition of a second biotin to biotin-PEG-3400 (biotin-PEG-biotin) resulted in a decrease in the inhibitory capacity, as reflected by its greater K_i value (19.08 μM). The reason for the reduced inhibitory ability of biotin-PEG-biotin is not clear at present and may be due to the difference in the nature/extent of its SMVT interaction. Biotin-PEG-3400 uptake in CHO/hSMVT cells was inhibited to similar extents by pantothenate, biocytin, desthiobiotin, and additional unlabeled biotin-PEG-3400, with maximal inhibition observed by biotin, which was in accordance with its low estimated K_i value (6.78 μM) in Caco-2 cells. Biocytin, a biotin analogue, was previously reported to be a weak inhibitor of SMVT-mediated biotin transport [32,36,38] due to the conversion of its valeric carboxyl group into an amide. Contrary to these findings, our results have indicated significant inhibitory abilities of biocytin in different cell systems expressing SMVT [20]. A probable reason for the contradictory observations is the incomplete characterization of the SMVT-inhibition process. For example, several compounds that lack a valeric carboxyl moiety, such as pantothenate and short chain fatty acids, have been shown to significantly inhibit the biotin-SMVT interaction [29,36,39]. In-depth

studies identifying the exact interactions between SMVT and its substrates are necessary to obtain conclusive evidence on its substrate requirements.

The transport of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ conjugate was concentration dependent, saturable and characterized by a low K_m (6.13 μM) similar to that of biotin-PEG-3400 (6.61 μM). The P_c of PEG-conjugated R.I-K(biotin)-Tat9 transport was greater than P_c of biotin-PEG-3400, but similar to the reported values for biotin (3.35 x 10^{-6} cm/sec) and R.I-K(biotin)-Tat9 [20 and Table 1, respectively]. In the control studies, the transport of PEG:(R.I-Cys-K-Tat9)$_8$ was very minimal (< 10^{-7} cm/sec) and not concentration dependent. The specific interaction of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ conjugate with SMVT was demonstrated using CHO/hSMVT cells. The J_{max} of conjugate uptake was 5.7-fold greater than the J_{max} of R.I-K(biotin)-Tat9 uptake (Table 1) [20]. In comparison, the uptake of PEG:(R.I-Cys-K-Tat9)$_8$ was very low (< 1 pmol/mg protein/10 min) in CHO/hSMVT and CHO/pSPORT cells. The uptake of the PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ was significantly ($p < 0.05$) inhibited by SMVT substrates biotin and pantothenate, biotin analogues desbiocytin and biocytin, and biotin-PEG-3400 and biotin-PEG-biotin.

During the transport PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ in Caco-2 and CHO cells, the peptide, R.I-Cys-K(biotin)-Tat9, was intracellularly cleaved from the PEG backbone due to the high reducing conditions. Since the tritium tag was located on the peptide (R.I-[^{3}H]- Cys-K(biotin)-Tat9), the intracellular transport of the PEG was not detectable. Further, the extent of intracellular cleavage of the 8 appended R.I-Cys-K(biotin)-Tat9 copies from the PEG could have varied between 0-8 for individual conjugate molecules, thereby confounding the quantification of conjugate transport. Therefore, the present

study reports the transport and uptake values of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ as a function of peptide concentration alone and not moles of conjugate. However, Low *et al.* have previously reported the cellular internalization of various biotinylated proteins, such as biotin-insulin, biotin-hemoglobin, and biotin-bovine serum albumin in suspended rat pheochromocytoma (PC-12), soybean, and carrot cells [40]. Although the study demonstrated the increased cellular uptake biotinylated compounds compared to the corresponding non-biotinylated controls, a mechanistic evaluation of the uptake process was lacking. While the authors implicated the role of receptor-mediated endocytosis through a transmembrane biotin receptor, the involvement of neither the biotin receptor nor the endocytotic uptake process was conclusively studied. Despite the inadequate mechanistic characterization, their studies demonstrate the utility of using biotinylated macromolecules for intracellular delivery.

In conclusion, we have characterized the transport properties of biotinylated PEG-based conjugates and demonstrated the specific involvement of SMVT. The present results suggest that the ability of high molecular weight conjugates with targeting moieties such as biotin to be transported efficiently across the intestinal membranes can potentially result in significant improvements in conventional drug delivery. Poorly absorbed compounds can potentially be conjugated to these polymeric carriers to enhance their intestinal absorption and oral bioavailability. Further, appending multiple copies of the therapeutic agent to the conjugate can increase the effective dose of drug delivered overcoming the potential limitation of inherently low capacity transporters.

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Notation

BOC: Tertiary-butyloxycarbonyl-

BOP/HOBt: (benzotriazolyl-oxy-tris-(dimethylamino)-phosphonium/hexafluoro phosphate /hydroxybenzotriazole)

DMF: Dimethylformamide

DIEA: N,N-diisopropylethylamine

DTT: Dithiothreitol

FBS: Fetal bovine serum

Fmoc: Fluorenylmethoxylcarbonyl-

NHS: Succinimide ester

R.I-K-Tat9: N-acetyl-D-Lys-D-Arg-D-Arg-D-Arg-D-Gln-D-Arg-D-Arg-D-Lys-D-Lys-D-Arg-NH_2

R.I-K(biotin)-Tat9: R.I-K-Tat9 with biotin linked to ε-N of N-terminal D-Lys

R.I-Cys-K(biotin)-Tat9: N-acetyl-D-Cys-D-Lys:(ε-biotin)-D-Arg-D-Arg-D-Arg-D-Gln-D-Arg-D-Arg-D-Lys-D-Lys-D-Arg-NH_2

SEC: Size-exclusion chromatography

SMVT: Sodium-dependent multivitamin transporter

SPDP: N-succinimidyl-3-(2-pyridylthio) proprionate

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TABLE

Table 1. Michaelis-Menten parameters for biotin-PEG-3400 and PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ transport across Caco-2 and CHO/hSMVT cells.

Cell System	Kinetic Parameter	Biotin-PEG-3400	PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ (moles of R.I-K(biotin)-Tat9)	R.I-K(biotin)-Tat9*

Caco-2 Cell Monolayer Transport	P_c (*10^{-6}) (cm/sec)	2.56 (0.11)	3.39 (0.53)	3.22 (0.30)
	P_m (*10^{-6}) (cm/sec)	1.61 (0.07)	2.18 (0.57)	0.57 (0.31)
	K_m (μM)	6.61 (1.25)	6.13 (3.74)	3.27 (1.46)
	J_{max} (μM.cm/sec)	----	2.31 (1.64) x 10^{-5}	1.19 (0.62) x 10^{-5}
CHO/ HSMVT Cell Uptake	J_{max} (pmol/mg protein/10 min)	23.46 (5.04)	1288.18 (54.25)	227.26 (29.33)
	K_m (μM)	1.26 (1.01)	8.19 (0.96)	1.00 (0.13)

P_c: Carrier-Mediated Permeability Component; P_m: Passive Permeability Component; J_{max}: Maximum Uptake Rate; K_m: Michaelis Constant. Numbers in parentheses represent Standard Deviation (S.D); n = 3. * Data from reference 13.

FIGURE CAPTIONS

1. Structure of the PEG:(RI-Cys-K(biotin)-Tat9)$_8$ conjugate, containing a bioreversible disulfide bond, used in the transport and uptake experiments

2. Absorptive transport of biotin-PEG-3400 (∇), PEG-3350 (Δ), and PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ (o) across Caco-2 cell monolayers. The experimentally observed values [Mean ± S.E.M] were used to plot the best-fit non-linear regression line (n = 3). Inset: Transformation of the conjugated R.I-K(biotin)-Tat9 transport data as a flux (J) versus concentrations (C) plot.

3. Uptake of biotin-PEG-3400 and PEG-3350 in CHO/hSMVT and CHO/pSPORT cells. Uptake was measured at pH 7.5 with 10-minute incubation (n = 3, mean ± S.E.M); (o) biotin-PEG-3400 uptake in CHO/hSMVT, (Δ) biotin-PEG-3400 uptake in CHO/pSPORT, (∇) PEG-3350 uptake in CHO/hSMVT. Inset: Transformation of the uptake data (for biotin-PEG-3400 into CHO/hSMVT cells) into an Eadie-Hofstee plot.

4. Lineweaver-Burke plots of biotin-PEG-3400 transport across Caco-2 cell monolayers in the presence of **A)** biotin, **B)** biocytin, and **C)** desthiobiotin, and D) biotin-PEG-biotin, used as inhibitors at 10 and 20 μM. The concentrations of the inhibitors and biotin-PEG-3400 in the apical solution are indicated on the regression lines and *x*-axis, respectively (n = 3).

5. Uptake of PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ in CHO/hSMVT cells (o), CHO/pSPORT cells (□) and PEG:(R.I-Cys-K-Tat9)$_8$ uptake in CHO/hSMVT cells (Δ). Uptake was measured at pH 7.5 with 10-minute incubation (n = 3, mean ± S.E.M).

6. Uptake of 0.1 μM PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ in CHO/hSMVT cells measured for 10 minutes in the presence of 50 μM of other known SMVT substrates. PEG:(R.I-Cys-K(biotin)-Tat9)$_8$ uptake is expressed as a percent of control (uptake of 0.1 μM biotin-PEG-3400 ~11.44 pmol/mg protein) (n = 4, mean ± S.E.M).

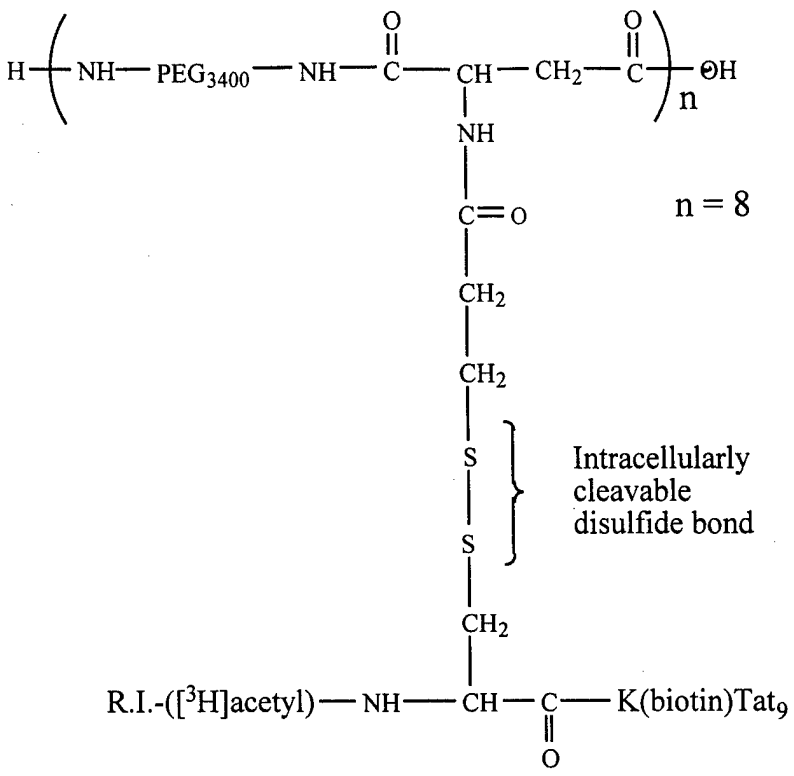
H
NH
PEG3400
NH
C
CH
CH2
C
OH
n
NH
C=O
n = 8
CH2
CH2
S
S
Intracellularly
cleavable
disulfide bond
CH2
R.I.-([3H]acetyl)
NH
CH
C
K(biotin)Tat9
O

Figure 1.

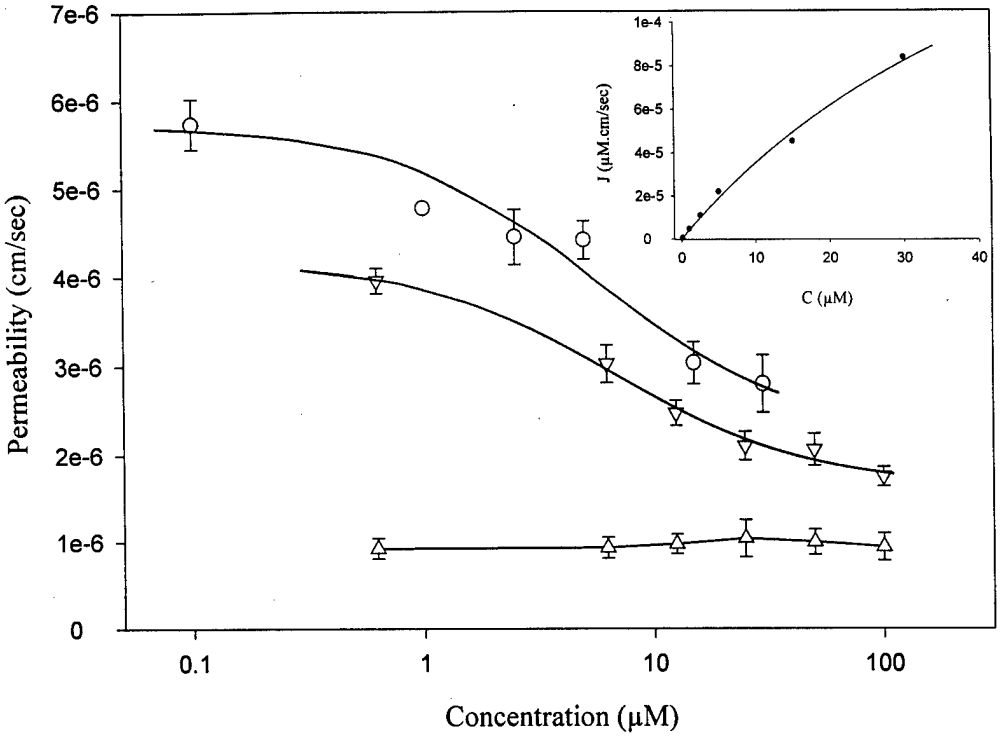
7e-6
6e-6
5e-6
4e-6
3e-6
2e-6
1e-6
0
Permeability (cm/sec)
0.1
1
10
100
Concentration (μM)
1e-4
8e-5
6e-5
4e-5
2e-5
0
J (μM.cm/sec)
0
10
20
30
40
C (μM)

Figure 2.

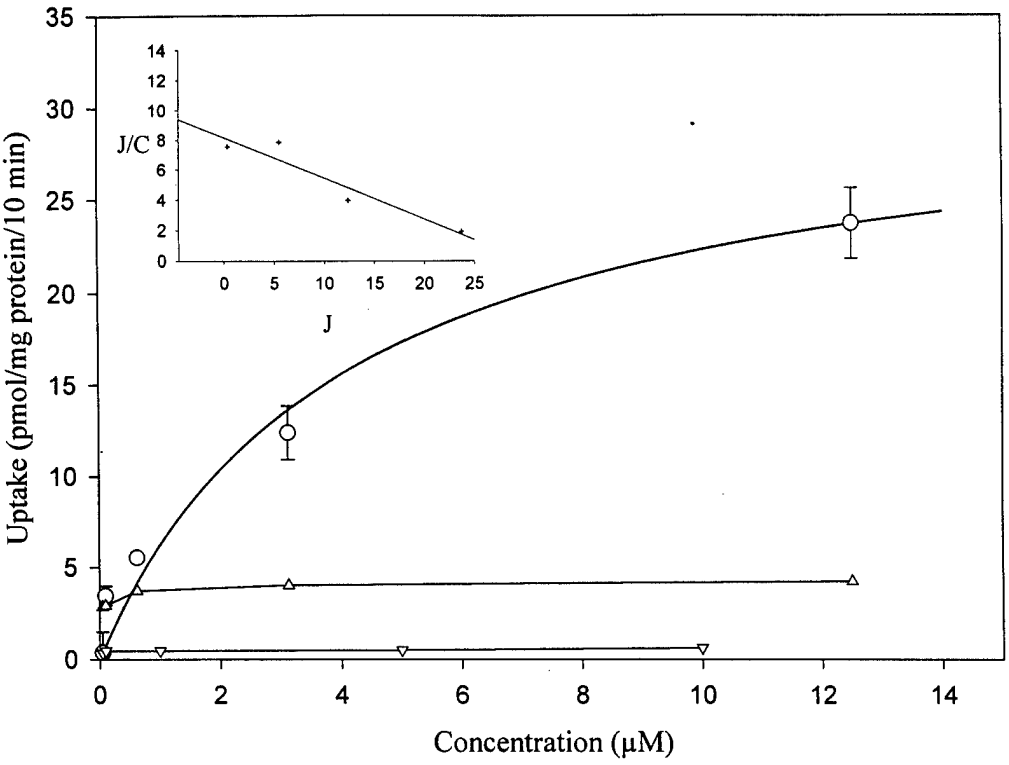
Uptake (pmol/mg protein/10 min)
Concentration (μM)
J/C
J
0
2
4
6
8
10
12
14
5
15
20
25
30
35

Figure 3.

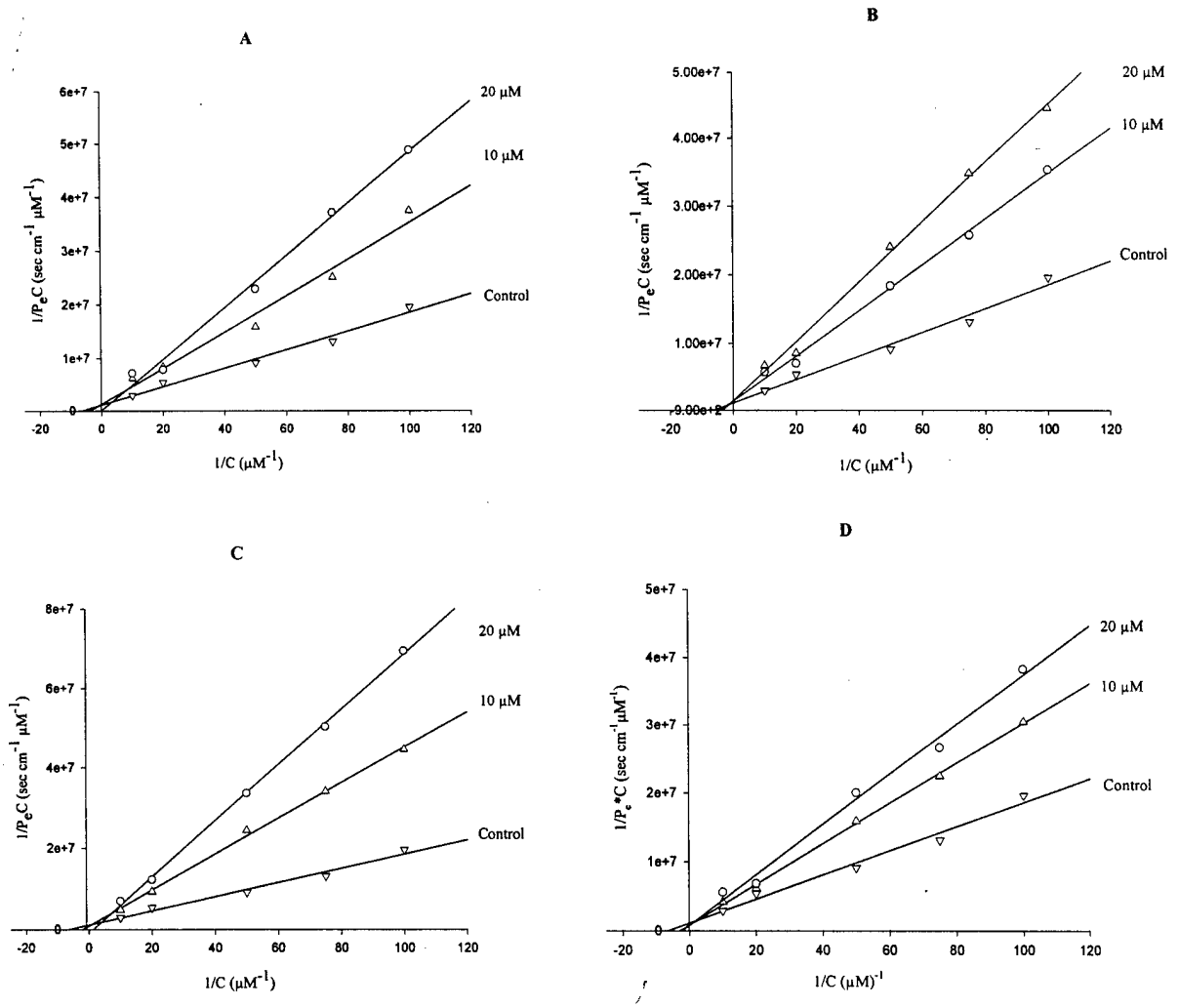
A
B
C
D
20 μM
10 μM
Control
1/C (μM⁻¹)
1/C (μM)⁻¹
1/Pe C (sec cm⁻¹ μM⁻¹)
1/Pe *C (sec cm⁻¹ μM⁻¹)

Figure 4.

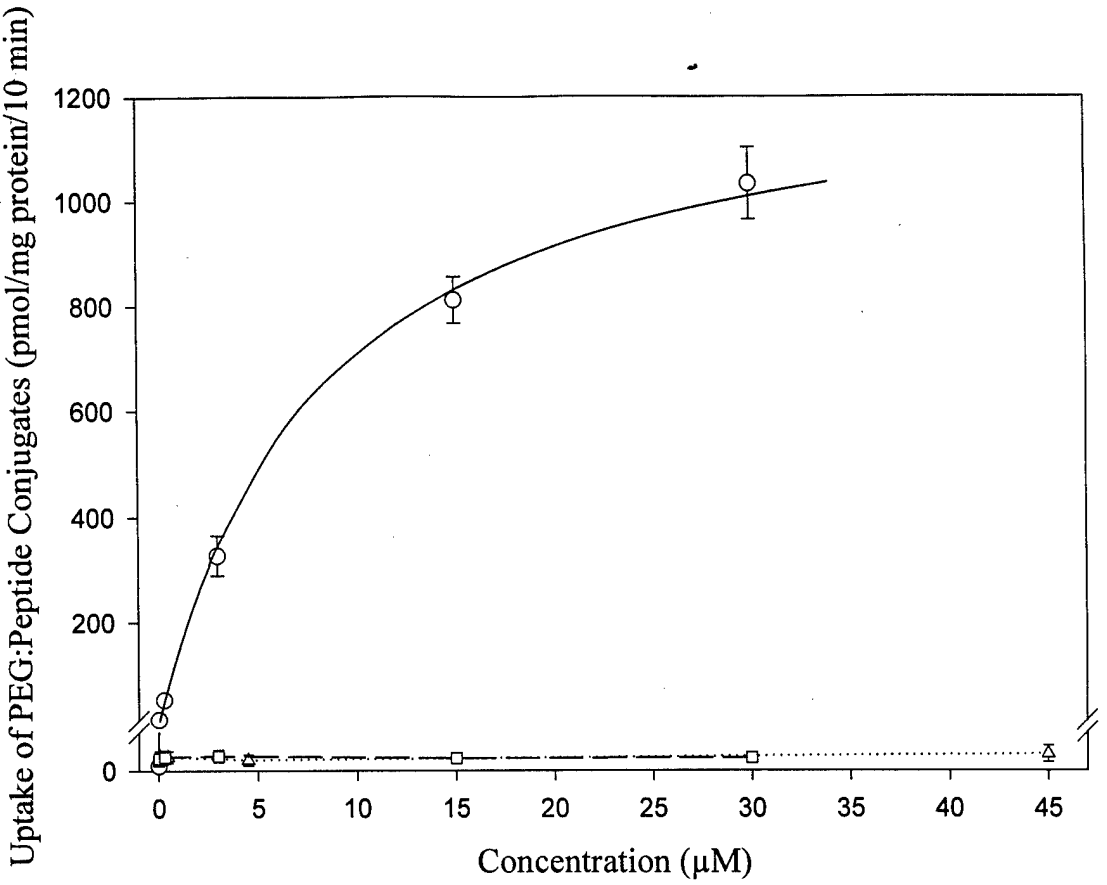
Uptake of PEG:Peptide Conjugates (pmol/mg protein/10 min)
1200
1000
800
600
400
200
0
0
5
10
15
20
25
30
35
40
45
Concentration (μM)

Figure 5.

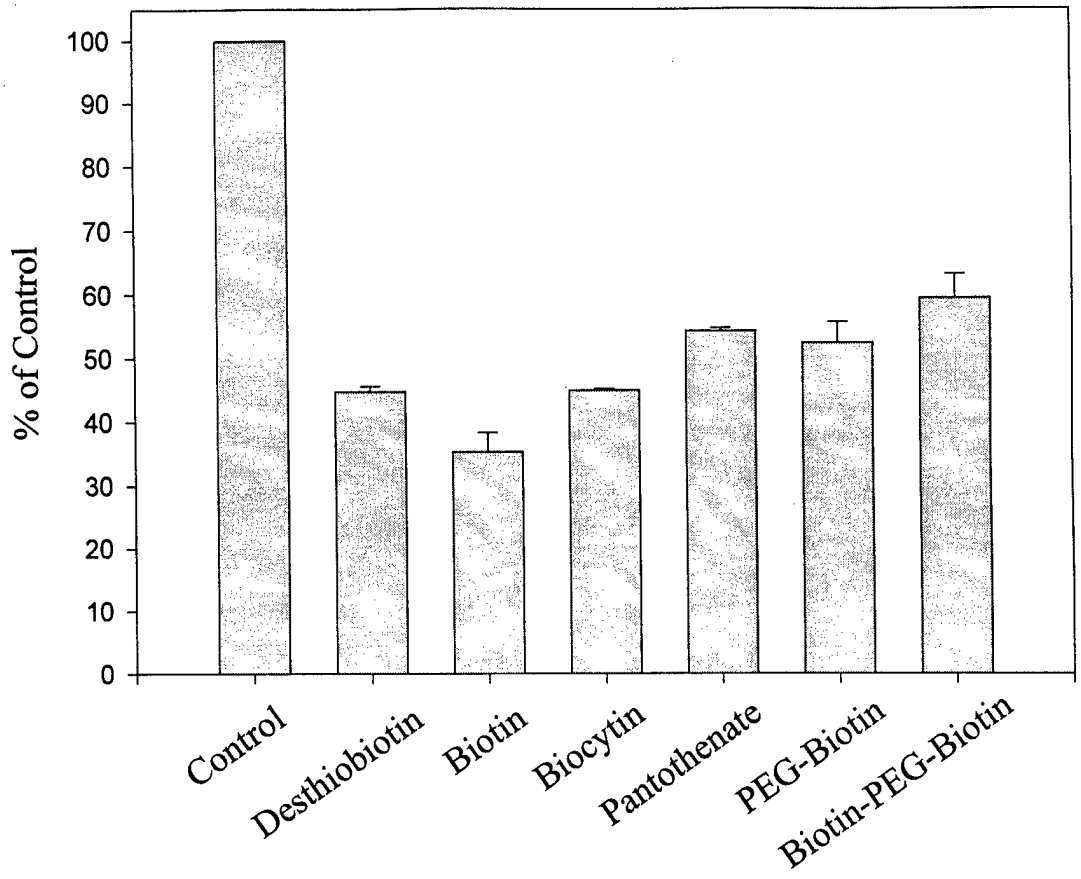
% of Control
100
90
80
70
60
50
40
30
20
10
0
Control
Desthiobiotin
Biotin
Biocytin
Pantothenate
PEG-Biotin
Biotin-PEG-Biotin

Figure 6.

The Department of Defense Breast Cancer Research Program Meeting

Era of Hope

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DUAL-SPECIFICITY ANTI-HER-2/NEU ANTISENSE DNA AGENTS FOR BREAST CANCER THERAPY

M. J. Leibowitz, J. Perlman, Q. Ding and S. Stein

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The HER-2/neu (erbB2) gene product is expressed at high levels in about one-third of breast cancers, and this aberrant expression correlates with poor prognosis. Reversal of this high level of expression should be a therapeutic measure that improves prognosis and response to other therapies. Antisense agents binding specifically to the 5' region of mRNA sensitize that region to digestion by endogenous RNase H, and thus reduce gene expression. Similarly, peptidomimetic agents binding to this region of mRNA can alter its expression. We propose that a dual specificity agent consisting of an antisense DNA linked to a peptidomimetic agent specifically binding to a nearby target on the HER-2/neu mRNA can result in enhanced selectivity and efficacy in degrading this specific RNA target.

Four different sequences have been reported for the DNA region 5' to the erbB2 gene. We found that only one of these yielded primers which allowed PCR amplification of this region, and sequence analysis of the product confirmed the correct sequence. We synthesized a fragment of 165 nucleotides of the 5' end of erbB2 mRNA, which is predicted to fold into a secondary structure resembling longer fragments of this RNA. Utilizing libraries of DNA oligonucleotides, we probed the secondary structure of this RNA to identify the sites of greatest sensitivity to RNase H cleavage. In parallel, we are synthesizing combinatorial libraries of peptidomimetic basic compounds on SPOTS membranes (Genosys), which we are screening for ability to specifically bind the 165 nucleotide RNA. Once we have identified an optimal target for RNase H cleavage and a peptidomimetic compound specifically binding to this RNA, we will link an antisense oligonucleotide complementary to the RNase H target to a peptidomimetic specifically binding to the RNA, to generate a dual-function agent. The ability of this agent to bind specifically to HER-2/neu mRNA and to inhibit expression of HER-2/neu will be tested in cultured breast cancer cells over-expressing this gene product.

The United States Army Medical Research and Materiel Command under DAMD17-97-1-7288 supported this work.

7th RCMI International Symposium Abstract Form

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Institution: Center for Advanced Biotechnology & Medicine

Bifunctional Antisense Agents Against HER-2/neu.

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UMDNJ-Robert Wood Johnson Medical School, Piscataway, N.J.

HER-2/neu is a member of the epidermal growth factor receptor family whose high level expression in some breast cancers and other malignancies correlates with poor prognosis. An antisense agent capable of down-regulating expression of this gene should be useful in treating these cancers. Inhibition of HER-2/neu expression by the therapeutic antibody Herceptin has been clinically useful in treating breast cancer. In order to design antisense agents with high affinity and specificity for the 5'-untranslated region (UTR) of HER-2/neu mRNA, we have designed bifunctional agents consisting of a short "active" DNA oligonucleotide binding to a sequence in this UTR, coupled to a "binding element" which is a chemical moiety with affinity for another portion of the UTR. The most active short DNA oligonucleotide tested was targeted to a predicted single stranded region of the UTR. We have produced bifunctional agents in which attachment of the binding element markedly stimulates RNase H sensitization at the site targeted by the active oligonucleotide. Experiments are in progress to test the ability of these bifunctional agents to down-regulate HER-2/neu in living cells.

Supported by Award DAMD17-97-1-7288 from the United States Army Medical Research and Materiels Command and IMSD Award 1R25 GM55145-04 from the National Institutes of Health.

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-OVER-

US006258774B1

(12) **United States Patent**
Stein et al.

(10) **Patent No.: US 6,258,774 B1**
(45) **Date of Patent: Jul. 10, 2001**

(54) **CARRIER FOR IN VIVO DELIVERY OF A THERAPEUTIC AGENT**

(75) Inventors: **Stanley Stein**, East Brunswick; **Michael J. Leibowitz**, Manalapan; **Patrick J. Sinko**, Lebanon, all of NJ (US)

(73) Assignee: **University of Medicine and Dentistry of New Jersey**, Piscataway, NJ (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/044,411**

(22) Filed: **Mar. 19, 1998**

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(52) **U.S. Cl.** **514/2**; 514/15; 525/50; 525/403; 530/327; 530/345; 530/408

(58) **Field of Search** 424/179.1, 180.1, 424/181.1, 194.1, 94.3; 435/188; 514/2, 15; 530/327, 328, 345, 391.3, 391.5, 391.7, 391.9, 404, 408; 525/50, 403, 430; 528/421, 491

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Grant. Hackh's Chemical Dictionary, 4th ed. New York:McGraw–Hill Book Co. p. 534, 1972.*
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(57) **ABSTRACT**

A carrier for in vivo delivery of a therapeutic agent comprising a thiol group is provided, wherein the therapeutic agent is conjugated to the carrier via a biodegradable disulfide bond. Since extracellular fluids in vivo do not provide the appropriate environment to efficiently reduce a disulfide bond, while cellular cytosol does provide an appropriate environment, the agent will remain substantially coupled to the carrier while circulating through the body until the carrier crosses a cell membrane. As a result, the therapeutic agent is protected from degradation and renal clearance, and the potential for the therapeutic agent to elicit an immune response is limited.

111 Claims, 7 Drawing Sheets

Scheme I

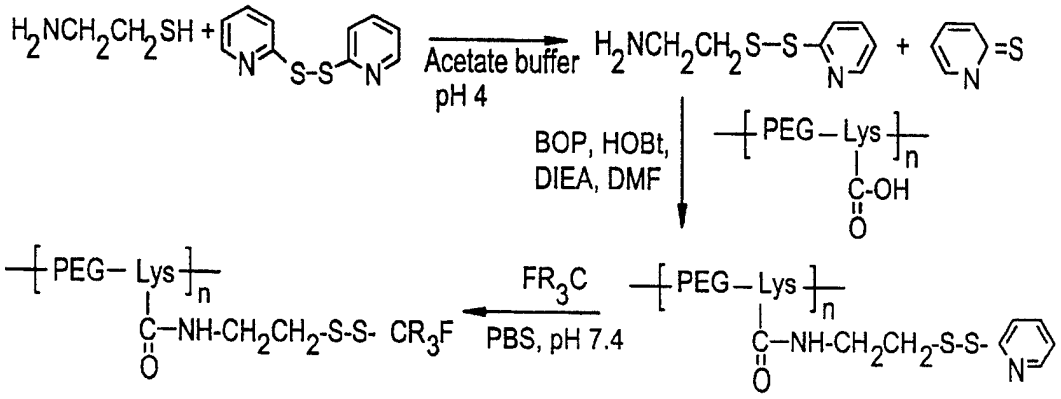


FIG. 1

Scheme I

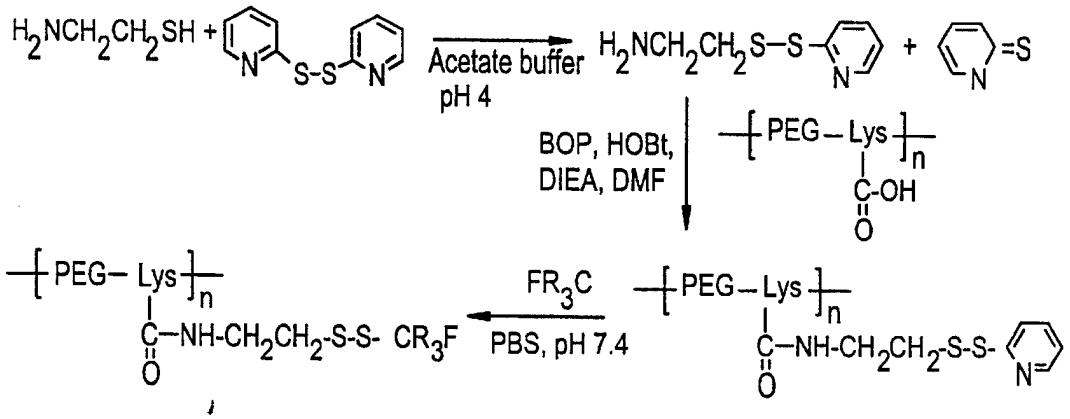


FIG. 2

Scheme II

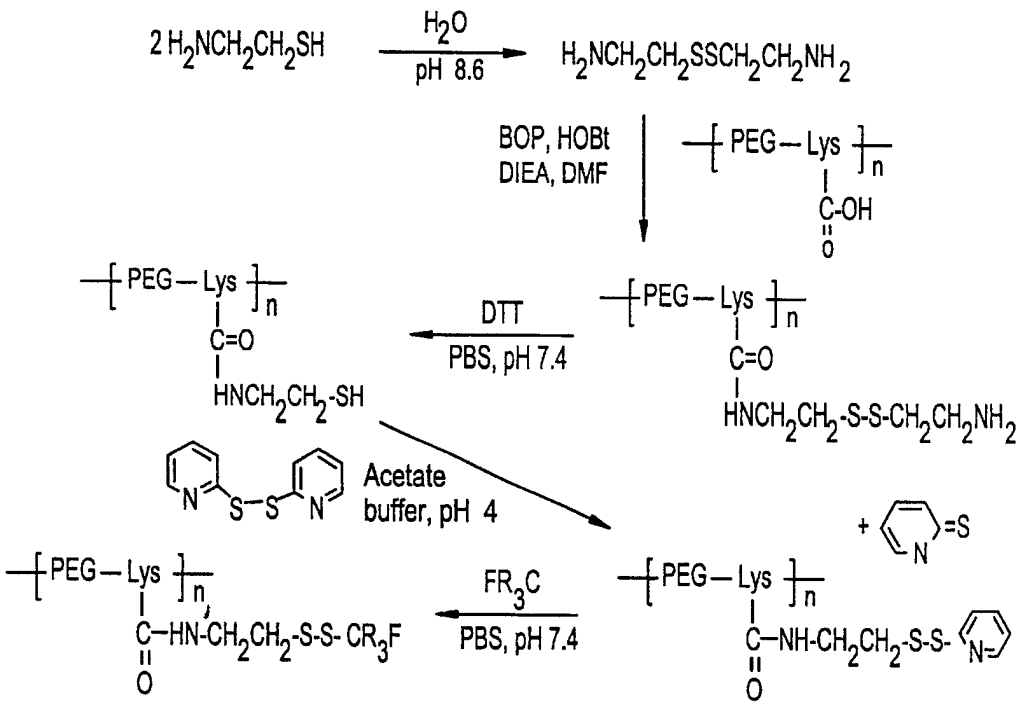


FIG. 3

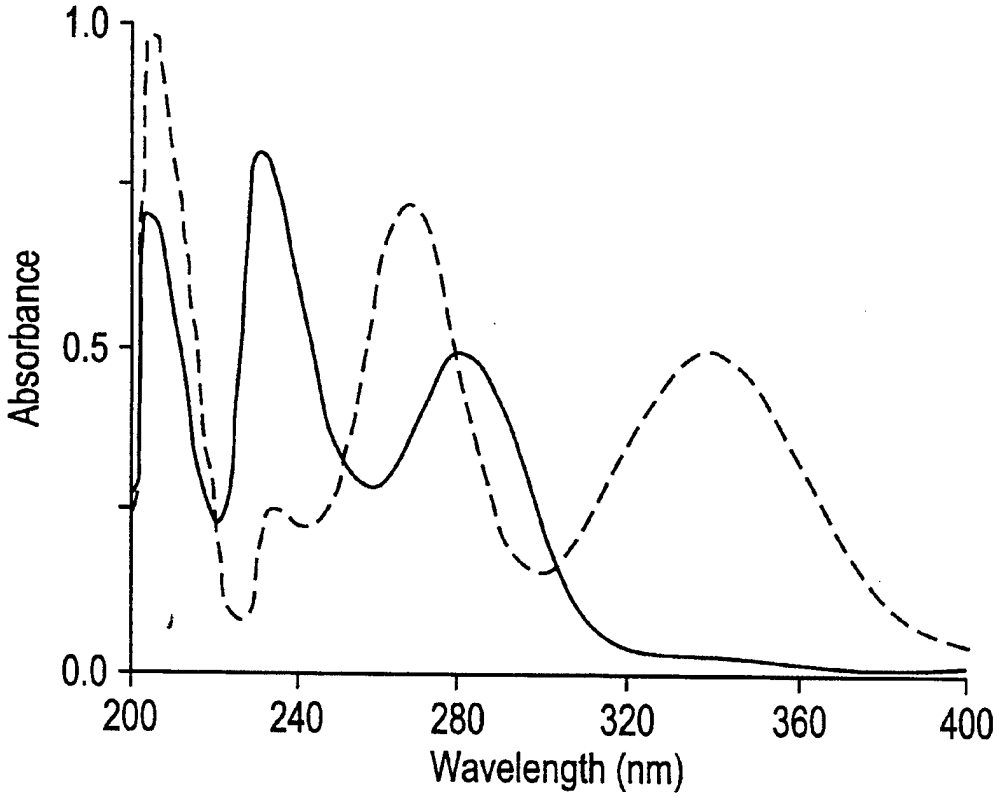


FIG. 4

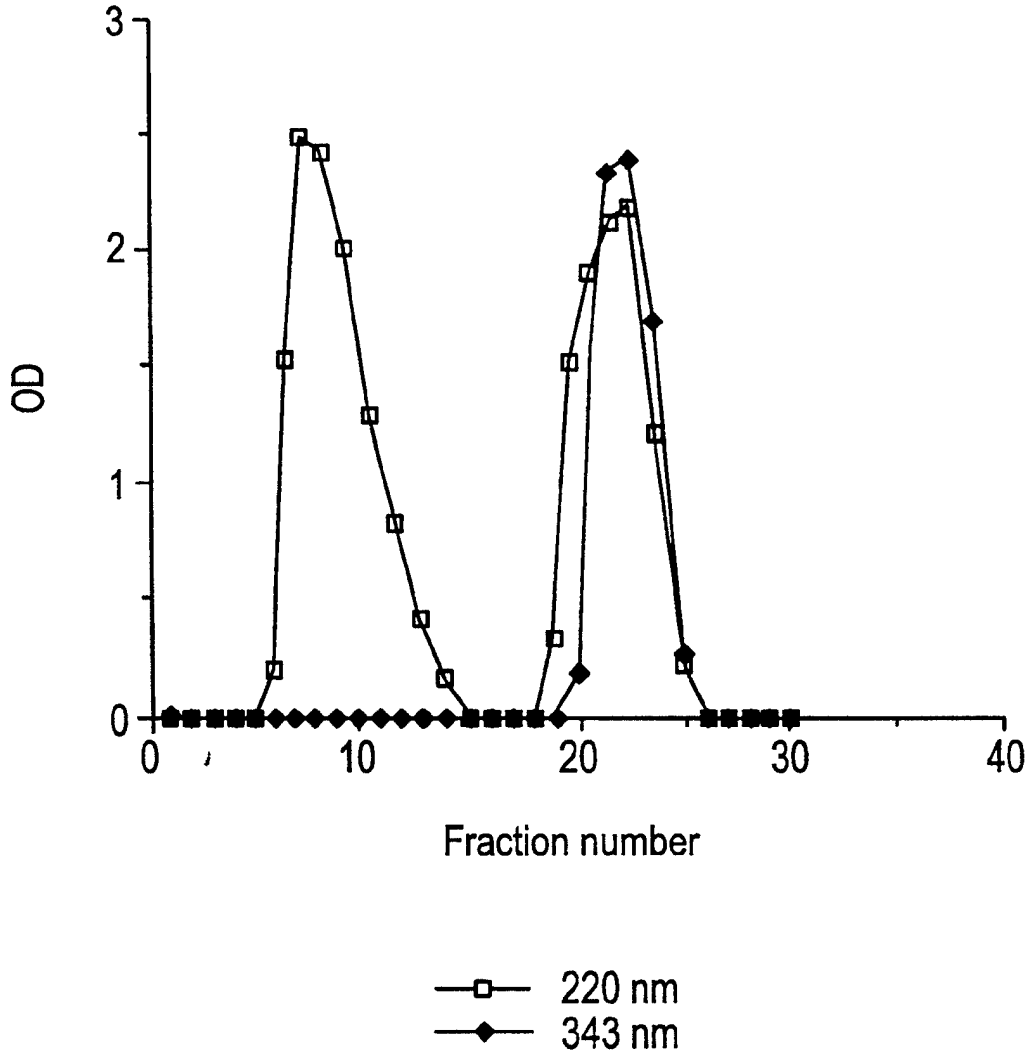


FIG. 5

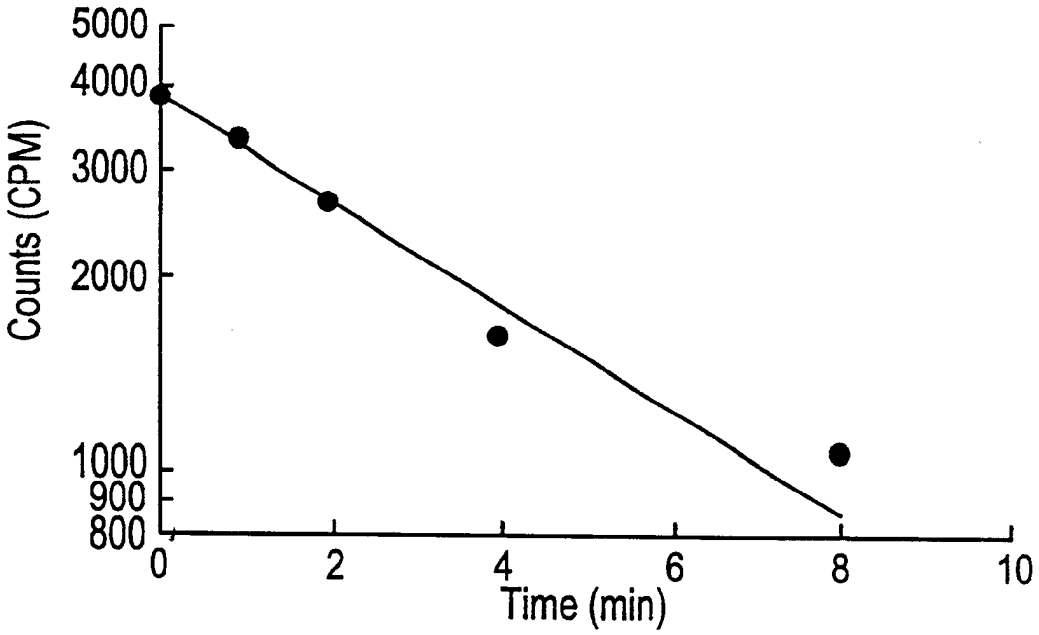


FIG. 6

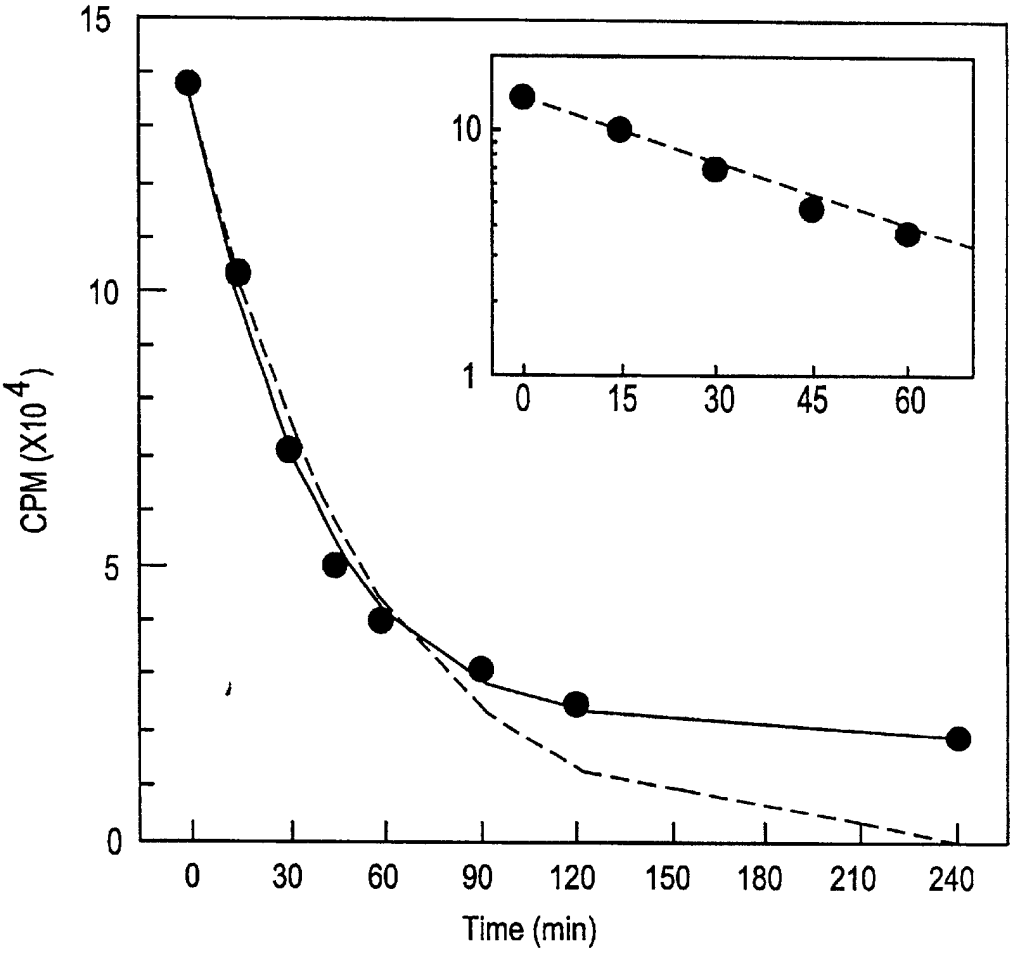
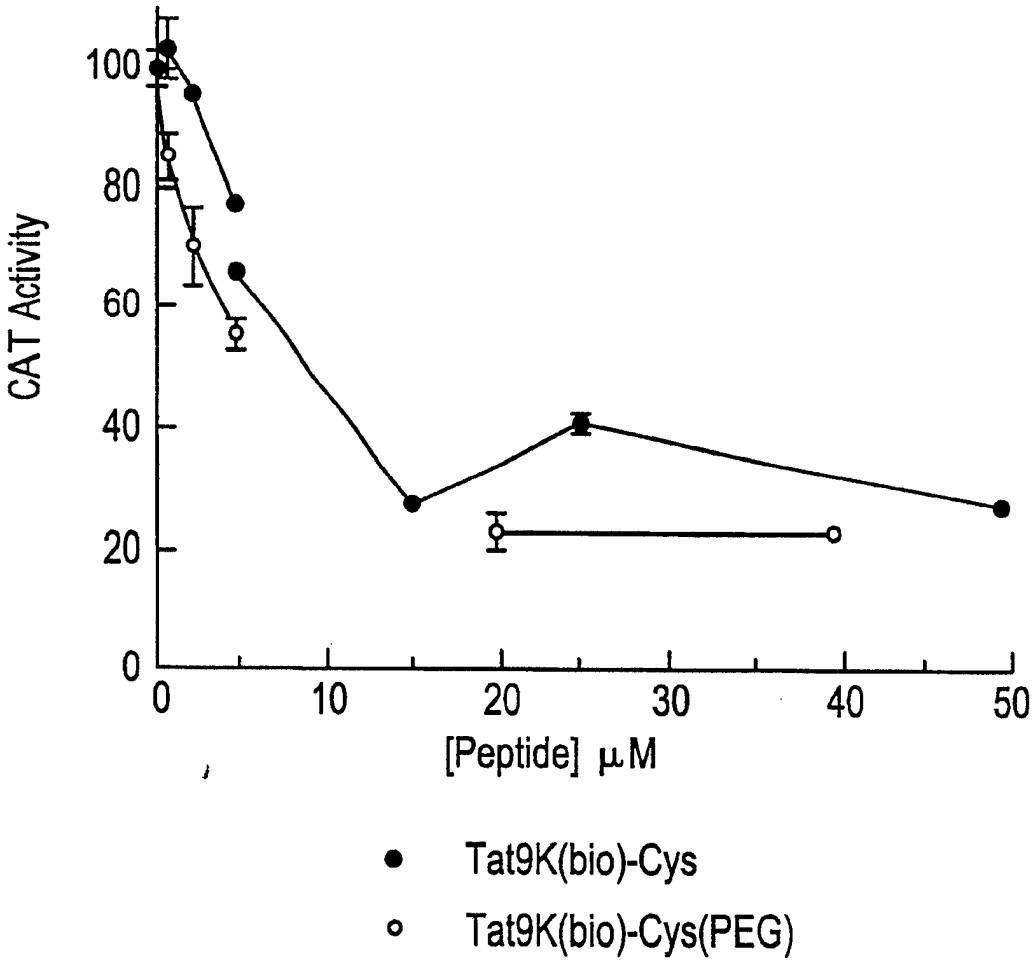

15
10
5
0
CPM (X10 4)
0
30
60
90
120
150
180
210
240
Time (min)
10
1
0
15
30
45
60

FIG. 7


CAT Activity
100
80
60
40
20
0
0
10
20
30
40
50
[Peptide] μM
Tat9K(bio)-Cys
Tat9K(bio)-Cys(PEG)

CARRIER FOR IN VIVO DELIVERY OF A THERAPEUTIC AGENT

FIELD OF THE INVENTION

The invention relates to chemical compounds for delivering therapeutic agents to tissues of a mammal, such as a human. More particularly, the invention involves a carrier for delivery of a therapeutic agent in which a biodegradable disulfide bond conjugates the therapeutic agent to a carrier of the present invention. The disulfide bond can be reduced in an appropriate environment in vivo so that a greater amount of the therapeutic agent is neither degraded nor excreted, but can be delivered to tissues, thus increasing its therapeutic effectiveness.

BACKGROUND OF THE INVENTION

It has been proposed that compounds, such as peptides, peptide mimetics, and oligonucleotides, or analogs or derivatives thereof, can be used as potential therapeutic agents. However, problems have been encountered in administering such compounds to a subject. For example, proteases and endonucleases present throughout the body digest such compounds, severely decreasing their biological activity. Other problems involve the elicitation of an immune response against the compound resulting in the degradation and inactivation of such compounds, and rapid renal clearance, particularly if the therapeutic agent has a low molecular weight. Hence, in order to be effective, such therapeutic agents must be administered frequently, and parenterally rather than orally. An example of such a therapeutic agent is insulin, which is typically injected several times daily by diabetics.

In efforts to overcome these problems, researchers have attempted to modify chemically such therapeutic agents in order to manipulate their pharmacologic properties[1], and perhaps enable them to survive longer in vivo before being degraded and removed from the blood stream. For example, one method of chemically modifying therapeutics is to append water-soluble polymer chains, such as polyethylene glycol (PEG), to the therapeutic agent[2]. Researchers have designed a PEG-lysine copolymer having multiple attachment sites[3], and have conjugated the copolymer to low molecular weight therapeutic agents. However, such modifications have inherent limitations. For example, they frequently interfere with the bioavailability of the therapeutic. Consequently, if the target for a therapeutic agent is intracellular, and the modification of the therapeutic prevents its crossing of the cell membrane, then the bioavailability of the therapeutic agent is reduced due to the chemical modification.

Another limitation to attaching a water-soluble polymer to a therapeutic agent involves modulating the biological activity of the therapeutic agent in a deleterious manner. For example, if the modification of the therapeutic agent alters its three dimensional structure, then its ability to bind a receptor site it was designed to bind can be decreased, resulting in a decrease of activity.

Hence, what is needed is a carrier of a therapeutic agent that reduces the chance of an elicitation of an immune response against the therapeutic agent.

Moreover, what is needed is a carrier that protects therapeutic agents from protease/peptidase/nuclease degradation in vivo, thereby eliminating the need for repetitive administration of the therapeutic agent.

In addition, what is needed is a carrier of a therapeutic agent that enhances cellular transmembrane delivery of the therapeutic agent.

Also, what is needed is a carrier of a therapeutic agent that does not release the therapeutic agent until the carrier has crossed the cell membrane, and once inside the cell, the carrier can release the therapeutic agent in a biologically active state.

What is also needed is a carrier of a therapeutic agent that does not interfere with the bioavailability of the therapeutic agent.

SUMMARY OF THE INVENTION

There is provided, in accordance with the present invention, a carrier for in vivo delivery of therapeutic agents that does not possess the shortcomings of other drug delivery carriers as described above, and offers the advantages of not interfering with the bioavailability of a therapeutic agent, protecting the therapeutic agent from proteolytic/nucleolytic degradation, from eliciting an immune response, and from rapid renal clearance, to name only a few.

Broadly, the present invention provides a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer, and at least one thiol compound conjugated to the polymer, such that the thiol group of the thiol compound and the thiol group of the therapeutic agent form a disulfide bond. The disulfide bond can be broken in a physiologically relevant reducing environment, such as that found in the cytosol of a cell. Hence, Applicants have discovered a way to modify a therapeutic agent to minimize degradation in vivo, and yet not limit its bioavailability. In addition, the present invention is particularly well suited to deliver therapeutic agents to the cytosol of cells. Glutathione, a naturally occurring reducing agent is found predominantly in cell cytosol. Hence, glutathione can reduce a disulfide bond, and release a therapeutic agent from a carrier of the present invention within the cells of the target tissue. In addition, since more than one thiol compound can be conjugated to the polymer, the carrier of the present invention can deliver more than one molecule of therapeutic agent to a target cell or tissue.

Numerous therapeutic agents comprise thiol groups, and can be used to conjugate the therapeutic agent to a carrier of the present invention with a disulfide bond. For example, therapeutic agents which are peptides comprising a cysteine residue can be delivered in vivo with a carrier of the present invention. In addition, analogs or derivatives of peptides which serve as therapeutic agents can be made to comprise a thiol group so that they can be delivered in vivo with a carrier of the present invention. Even nucleotides and analogs or derivatives thereof, used in antisense therapy for example, can be easily modified to comprise a thiol group in order to be carried via a carrier of the present invention.

Another example of a therapeutic agent comprising a thiol group, which can be conjugated to a a carrier of the present invention for in vivo delivery, is a therapeutic agent which inhibits HIV-1 replication. More specifically, it has been determined that the HIV Tat protein strongly activates HIV transcription through its interactions with the TAR RNA region. The TAR RNA domain consists of the first 57 nucleotides of all virally encoded RNAs. The predicted TAR RNA secondary structure is a double-stranded stem with a 3-base bulge and a 6-base loop. HIV-1 Tat is a small nuclear protein containing 86–102 amino acids, and is encoded by multiply spliced mRNA. The 3-base bulge in TAR RNA and several other flanking nucleotides are essential for Tat-TAR interaction.

Tat protein apparently acts to promote transcription by binding through its basic domain to the 3-base bulge of TAR.

This is accompanied by recruitment of host cellular factors, including Tat and TAR binding proteins, to the TAR RNA stem and 6-base loop, as well as to the complex of template DNA, transcription factors and RNA polymerase. Initiation of proviral gene expression appears to occur by activation of an NF-κB and/or Sp1-dependent promoter, resulting in production of viral transcripts at a sufficient level to provide synthesis of Tat protein, which then interacts with TAR to allow enhanced production of elongated HIV transcripts.

Efforts have been made to develop a therapeutic agent which binds TAR, and blocks Tat-TAR binding. For example, a 10-residue Tat peptide with an appended 4-mer antisense oligonucleotide can specifically bind to TAR RNA, as shown by its ability to stimulate RNase H-mediated cleavage at the site of oligonucleotide annealing to the 6-base single-stranded loop. In another example, a biotinylated peptide has also been shown to inhibit Tat binding to TAR (please see Choudhury, I., Wang, J., Rabson, A. B., Stein, S., Pooyan, S., Stein, S. and Leibowitz, M. J. (1998) Inhibition of HIV-1 replication by a Tat RNA binding domain peptide analog. *J. Acqu. Immune Def. Syndr. & Human Retrovirol.*, 17, 104–111, incorporated by reference herein in its entirety).

Hence, the present invention extends to a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer, and at least one thiol compound conjugated to the polymer, such that the thiol group of the thiol compound and the thiol group of the therapeutic agent form a disulfide bond, and the therapeutic agent is a Tat inhibitory polypeptide derivative. More particularly, in an embodiment of the invention, a therapeutic agent comprising a thiol group, which is a Tat-inhibitory binding peptide derivative, relates to biotinylated peptides of the formula I:

R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-Cys-NH_2 (SEQ ID NO: 1)

wherein R is the residue of the carboxylic acid, or an acetyl group, and X is a Cys or Lys residue, and analogs thereof, and biologically and pharmaceutically acceptable salts thereof, all stereo, optical and geometrical isomers thereof where such isomers exist, as well as the pharmaceutically acceptable salts and solvates thereof, which exhibit advantageous properties, including binding to ΔTAR, inhibition of LTR-dependent reporter gene expression in a model cell assay and, finally, inhibition of HIV-1 replication, as determined as assays of HIV-induced syncytium formation, cytotoxicity and reverse transcriptase production. The biotinylated peptide of formula I can be readily conjugated to a carrier of the present invention via a disulfide bond between the sulfur of the thiol group of a Cys residue and the sulfur of the thiol group of the thiol compound of the carrier. Examples of such Tat peptide derivatives include, but are not limited to:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:2)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:4)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:5)

N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:6)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys (biotin)-Cys-NH_2 (SEQ ID NO:7).

In another embodiment, the present invention extends to a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer, and at least one thiol compound conjugated to the polymer, such that the thiol group of the thiol compound and the thiol group of the therapeutic agent form a disulfide bond, and the therapeutic agent is a Tat inhibitory polypeptide derivative comprising an amino acid sequence of:

N-acetyl-DCys-DLys-(biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-NH_2 (SEQ ID NO:8)

or analogs or derivatives thereof, and biologically and pharmaceutically acceptable salts thereof, which exhibit advantageous properties, including binding to ΔTAR, inhibition of LTR-dependent reporter gene expression in a model cell assay and, finally, inhibition of HIV-1 replication.

Furthermore, the present invention extends to a method of treating a viral infection in a mammal in need of such treatment. More particularly, such a method comprises administering to a mammal a therapeutically effective amount of a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer, and at least one thiol compound conjugated to the polymer, such that the thiol group of the thiol compound and the thiol group of the therapeutic agent form a disulfide bond, and the therapeutic agent comprises a Tat-inhibitory binding peptide derivative which comprises a biotinylated peptide of the formula I:

R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-Cys-NH_2 (SEQ ID NO:1)

wherein R is the residue of a carboxylic acid or acetyl group, X is a Cys or Lys residue, analogs thereof, and biologically and pharmaceutically acceptable salts thereof. Throughout the specification and appended claims, the polypeptide of formula I, and its analogs and salts, encompass all stereo, optical and geometrical isomers thereof where such isomers exist, as well as the pharmaceutically acceptable salts and solvates thereof. Where appropriate, the polypeptide or its analogs can be utilized as its corresponding amide form. The amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide.

More particularly, a therapeutic agent having applications in such a method comprises an amino acid sequence including, but not limited to:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:2)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO: 3)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:4)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO.-5)

N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:6)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys (biotin)-Cys-NH_2 (SEQ ID NO:7).

In another embodiment, the present invention extends to a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer, and at least one thiol compound conjugated to the polymer, such that the thiol group of the thiol compound and the thiol group of the therapeutic agent form a disulfide bond, and the therapeutic agent is a Tat inhibitory polypeptide derivative comprising an amino acid sequence as set forth below:

N-acetyl-DCys-DLys-(biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-NH_2 (SEQ ID NO:8)

or analogs or derivatives thereof, and biologically and pharmaceutically acceptable salts thereof, which exhibit advantageous properties, including binding to ΔTAR, inhibition of LTR-dependent reporter gene expression in a model cell assay and, finally, inhibition of HIV-1 replication.

Moreover, the present invention extends to a method of utilizing a carrier of the present invention conjugated to a peptide comprising an amino acid sequence as set forth in SEQ ID NO:8, and biologically and pharmaceutically acceptable salts thereof, to treat a viral infection in a mammal in need of such treatment. Such a method comprises administering to a mammal a therapeutically effective amount of a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer, and at least one thiol compound conjugated to the polymer, such that the thiol group of the thiol compound and the thiol group of the therapeutic agent form a disulfide bond, and the therapeutic agent comprises a biotinylated peptide comprising an amino acid sequence as set forth in SEQ ID NO:8.

In particular, methods of the present invention stated above can be used to treat retroviral infections such as AIDS, in humans.

Moreover, the invention extends to a carrier for in vivo delivery of a therapeutic agent, comprising a polymer which has a branched or linear structure. For purposes of this application, the term "polymer" encompasses both homopolymers and copolymers. Preferably, the polymer is a water soluble polymer.

Examples of water soluble polymers which have applications in the present invention include, but are not limited to, polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, polyaminoacids (homopolymers), polypropylene glycol, copolymers of ethylene glycol/propylene glycol, ethylenelmaleic anhydride copolymer, polyaminoacids, copolymer of polyethylene glycol and an amino acid, polypropylene oxide/ethylene oxide copolymers, or polyethylene glycol/thiomalic acid copolymers. In a preferred embodiment, the polymer comprises a copolymer of polyethylene glycol and lysine.

Moreover, a thiol compound can be conjugated to the polymer of the carrier, or conjugated to at least one functional group attached to the polymer which is available for reaction with the thiol compound, provided the thiol group of the thiol compound is available to form a disulfide bond with a thiol group of the therapeutic agent.

Numerous functional groups can be attached to a polymer of a carrier of the present invention and used to conjugate a thiol compound thereto. Moreover, more than one type of functional group can be concurrently attached to the polymer, and available to conjugate a thiol compound to the polymer. Examples of functional groups having applications in this embodiment of the present invention include, but are not limited to, ketones, esters, carboxylic acids, aldehydes, alcohols, thiols, or amines. In a preferred embodiment, the polyethylene glycol/lysine copolymer has a carboxylic acid group attached thereto, and available for reaction. Hence, more than one molecule of therapeutic agent can be conjugated to a carrier of the present invention, and delivered to a target cell or tissue.

A polymer of a carrier of the present invention can have any molecular weight. In an embodiment of the invention, the polymer has a molecular weight range of about 1,000 to about 1,000,000 Daltons, and preferably a molecular weight range of about 20,000 to 200,000 Daltons. In a preferred embodiment, the polymer has a molecular weight of about 27,000 Daltons.

The present invention further extends to a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer, and at least one thiol compound conjugated to the polymer at an interval so that the thiol group of the at least one thiol compound and a thiol group of a therapeutic agent can form a disulfide bond. As used herein, the term "interval" indicates a distance between thiol compounds conjugated to the polymer of a carrier of the present invention. In an embodiment, the interval between thiol compounds conjugated to the polymer is about 100 to about 10,000 Daltons. In a preferred embodiment, the interval between conjugation of thiol compounds to a polymer of the carrier is about 300 to about 3,000 Daltons.

Furthermore, the present invention extends to a carrier comprising a polymer with functional groups attached thereto, wherein the functional groups are available for conjugation to a thiol compound, and are attached to the polymer at an interval. In an embodiment, the interval between functional groups attached to the polymer is about 100 to about 10,000 Daltons. In a preferred embodiment, the interval between functional groups attached to a polymer of the carrier is about 300 to about 3,000 Daltons.

Moreover, examples of thiol compounds having applications in a carrier of the present invention include, but are not limited to, cysteamine, 1-amino-2-methyl-2-propanethiol, or 1-amino-2-propanethiol, to name only a few.

In addition, the present invention extends to a carrier for in vivo delivery of a therapeutic agent having a thiol group, wherein the carrier further comprises a cell uptake promoter conjugated to the polymer. The cell uptake promoter enhances the ability of the carrier with the therapeutic agent conjugated thereto, to cross a cell membrane and enter the cell's cytosol.

Numerous cell uptake promoters are known, and have applications in embodiments of the present invention. An example of a cell uptake promoter having applications therein is biotin. Hence, conjugation of biotin to a polymer of a carrier of the present invention enhances the ability of the carrier, and the therapeutic agent attached thereto via a disulfide bond, to cross a cell membrane and enter a cell's cytosol. Once inside the cell, the disulfide bond between the therapeutic agent and the carrier is reduced, and the therapeutic agent is released to act upon its target.

Moreover, it has been discovered that the cell uptake promoter need not be conjugated only to the polymer in order to be effective. Rather, the cell uptake promoter can also be conjugated to the therapeutic agent, and still enhance the ability of the carrier and the therapeutic agent to cross a cell membrane.

The present invention further extends to methods of making a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer and at least one thiol compound conjugated to the polymer, such that the thiol group of the thiol compound and the thiol group of the therapeutic agent form a disulfide bond. One such method disclosed herein comprises the steps of:

a) reacting a thiol compound with a disulfide compound to form a first intermediate wherein the thiol group of the thiol compound and a sulfur atom of the disulfide bond of the disulfide compound form a disulfide bond; and

b) reacting the first intermediate with a polymer to form the carrier, wherein the first intermediate is conjugated to the polymer, so that the sulfur atom of the thiol compound of the carrier and the thiol group of the therapeutic agent can form a disulfide bond.

Any disulfide compound can be used in step (a) of the method recited above. Preferably, the disulfide compound is symmetric. In an embodiment of the present invention, the disulfide compound is 2,2'-dithiodipyridine.

Another method of making a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, as set forth herein, comprises the steps of:

a) reacting a first thiol compound with a second thiol compound to form a first intermediate comprising a disulfide bond;

b) reacting the first intermediate with a polymer to form a second intermediate, wherein the first intermediate is conjugated to the polymer;

c) reducing the disulfide bond to form a third intermediate comprising the polymer and the first thiol compound conjugated to the polymer so that the thiol group of the first thiol compound is available for reaction; and

d) reacting the third intermediate with a disulfide compound to form the carrier, wherein the thiol group of the first thiol compound and a sulfur atom of the disulfide bond of the disulfide compound form a disulfide bond.

Under appropriate conditions, the sulfur atom of the first thiol compound and the thiol group of the therapeutic agent can form a disulfide bond.

In an embodiment of the present invention, the first thiol compound and the second thiol compound are the same compound.

Numerous thiol compounds have applications in methods of the present invention. Examples include cysteamine, t-amino-2-methyl-2-propanethiol, and 1-amino-2-propanethiol, to name only a few.

Moreover, any disulfide compound can be used in step (d) of this method. Preferably, the disulfide compound is symmetric. In an embodiment of the present invention, the disulfide compound is 2,2'-dithiodipyridine.

In an embodiment of the present invention, a therapeutic agent comprising a thiol group is a Tat-inhibitory peptide derivative, and comprises a biotinylated peptide of the formula I:

R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-Cys-NH_2 (SEQ ID NO:1)

wherein R is the residue of a carboxylic acid or an acetyl group, and X is a Cys or Lys residue, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof, and all stereo, optical and geometrical isomers thereof where such isomers exist, as well as the pharmaceutically acceptable salts and solvates thereof. Examples of analogs of such a therapeutic agent include, but are not limited to:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:2)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:4)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:5)

N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:6)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys (biotin)-Cys-NH_2 (SEQ ID NO:7).

In another embodiment of the present invention, a therapeutic agent comprising a thiol group is a Tat-inhibitory peptide derivative comprising an amino acid sequence of SEQ ID NO:8:

N-acetyl-DCys-DLys-(biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-NH_2 (SEQ ID NO:8)

analogs or derivatives thereof, as well as all pharmaceutically acceptable salts thereof.

The production of such agents can readily be accomplished with presently known method of producing peptide including, but not limited to solid phase synthesis of peptides. Moreover, it is also readily apparent to one skilled in the art on methods of biotinylating such peptides.

The present invention further extends to methods of making a carrier for in vivo delivery of a therapeutic agent, as set forth above, wherein the polymer has a branched or linear structure. Preferably, the polymer is a water soluble polymer. Examples of water soluble polymers having applications herein include polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, polyaminoacids (homopolymers), polypropylene glycol, copolymers of ethylene glycol/propylene glycol, ethylene/maleic anhydride copolymer, polyaminoacids, copolymer of polyethylene glycol and an amino acid, polypropylene oxide/ethylene oxide copolymers, or polyethylene glycol/thiomalic acid copolymers.

Moreover, a polymer used in methods of the present invention can have any molecular weight. In an embodiment of the present invention, a polymer has a molecular weight range of about 1,000 to about 1,000,000 Daltons, and preferably a molecular weight range of about 20,000 to 200,000 Daltons. In a preferred embodiment, the polymer has a molecular weight of about 27,000 Daltons. The present invention also extends to methods of making a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, comprising a polymer and at least one thiol compound conjugated to the polymer at an interval. In an embodiment of the invention, the interval between thiol compounds conjugated to the polymer is about 100 to about 10,000 Daltons, and preferably about 300 to about 3,000 Daltons.

The present invention further extends to methods for making carriers for in vivo delivery of a therapeutic agent comprising a thiol group, further comprising the step of conjugating a cell uptake promoter to the polymer prior to reacting the polymer with an intermediate. The cell uptake promoter enhances the ability of the carrier bound to the therapeutic agent to cross a cell membrane and enter the cell's cytosol.

Numerous cell uptake promoters are known, and have applications in the present invention. An example of a cell uptake promoter is biotin. Hence, conjugating biotin to a polymer of a carrier of the present invention enhances the ability of the carrier and the therapeutic agent conjugated thereto, to cross a cell membrane and enter a cell's cytosol. As explained above, once the carrier crosses the cell membrane and enters the cytosol, the disulfide bond is reduced, and agent is released to act on its target.

Moreover, it has been discovered that the cell uptake promoter need not be attached only to the polymer. Rather, the cell uptake promoter can also be conjugated to the therapeutic agent, and still enhance the ability of the carrier and the therapeutic agent to cross a cell membrane.

The present invention further extends to a method of making a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer comprising at least one functional group attached thereto, and a thiol compound conjugated to the at least one functional group, such that the thiol group of the thiol compound and the thiol group of the therapeutic agent form a disulfide bond. An example of a method for making such a carrier comprises the steps of:

a) reacting a thiol compound with a disulfide compound to form a first intermediate wherein the thiol group of the thiol compound and a sulfur atom of the disulfide bond of the disulfide compound form a disulfide bond; and

b) reacting the first intermediate with a polymer comprising at least one functional group attached thereto to form the carrier, wherein the first intermediate is conjugated to the functional group so that the sulfur atom of the thiol compound and the thiol group of the therapeutic agent can form a disulfide bond under appropriate conditions.

Furthermore, the present invention extends to a method of making a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, as set forth herein, comprising the steps of:

a) reacting a first thiol compound with a second thiol compound to form a first intermediate, wherein the thiol group of the first thiol compound and the thiol group of the second thiol compound form a disulfide bond;

b) reacting the first intermediate with a polymer comprising at least one functional group attached thereto to form a second intermediate, wherein the first intermediate is conjugated to the at least one functional group;

c) reducing the disulfide bond of the second intermediate to form a third intermediate comprising the polymer with at least one functional group and the first thiol compound conjugated to the functional group, so that the thiol group of the first thiol compound is available for reaction;

d) reacting the third intermediate with a disulfide compound to form the carrier, wherein the thiol group of the first thiol compound and a sulfur atom of the disulfide bond of the disulfide compound form a disulfide bond. Hence, under appropriate conditions, the disulfide bond of the carrier can be reduced, and the sulfur atom of the first thiol compound and the thiol, group of the therapeutic agent can form a disulfide bond.

In an embodiment of the present invention, the first thiol compound and the second thiol compound can be the same compound.

The present invention extends to methods of making a carrier of the present invention, as set forth above, wherein the at least one functional group attached to a polymer used in methods of the present invention comprises a ketone, an ester, a carboxylic acid, an aldehyde, an alcohol, a thiol, or an amine, to name only a few.

Moreover, the present invention extends to methods of making a carrier of the present invention as described above, wherein the at least one functional group is attached to the polymer of a carrier of the present invention at an interval. In an embodiment of the invention, the interval between functional groups attached to the polymer is about 100 to about 10,000 Daltons, and preferably about 300 to about 3,000 Daltons.

As stated above, a therapeutic agent conjugated to a carrier of the present invention comprises at least one thiol group. In an example, a therapeutic agent conjugated to a carrier of the present invention comprises a Tat inhibitory peptide derivative of the formula I:

R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-Cys-NH_2 (SEQ ID NO: 1)

wherein R is the residue of a carboxylic acid or an acetyl group, and X is a Cys or Lys residue, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof, along with all stereo, optical and geometrical isomers thereof, where such isomers exist, and pharmaceutically acceptable salts and solvates thereof.

Moreover, examples of analogs of such a therapeutic agent having applications in the present invention include, but are not limited to:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:2)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:4)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:5)

N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:6)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:7),

to name only a few.

In another embodiment, a therapeutic agent comprising a thiol group and conjugated to a carrier of the present invention, comprises an amino acid sequence as set forth in SEQ ID NO:8:

N-acetyl-DCys-DLys-(biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-NH_2 (SEQ ID NO:8).

or analogs or derivatives thereof, and pharmaceutically acceptable salts thereof.

Applicants have also discovered that the release rate of a therapeutic agent from a carrier of the present invention can be modulated, depending on the steric hindrance of the thiol compound of the carrier. In particular, Applicants have discovered that the greater the steric hindrance of the thiol compound, the slower the release rate of the therapeutic agent from the carrier. Hence, the present invention extends to a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer and at least one thiol compound comprising at least one functional group, conjugated to the polymer, such that the thiol group of the thiol compound and the thiol group of the therapeutic agent can form a disulfide bond, and the rate at which the disulfide bond is reduced in an appropriate physiological environment is dependent on the size of the at least one functional group attached to the thiol compound.

Moreover, the present invention extends to methods of making a carrier of the present invention as described above, wherein the at least one functional group is attached to the polymer of a carrier of the present invention at an interval. In an embodiment of the invention, the interval between functional groups attached to the polymer is about 100 to about 10,000 Daltons, and preferably about 300 to about 3,000 Daltons.

The present invention further extends to another embodiment wherein the release rate of a therapeutic agent from a carrier of the present invention can be modulated depending on the steric hindrance of the thiol compound of the carrier. In particular, disclosed herein is a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer and at least one thiol compound comprising at least one functional group, and a polymer comprising at least one functional group available for reaction, so that the thiol compound is conjugated to the at least one functional group attached to the polymer, and the thiol group of the thiol compound and the thiol group of the therapeutic agent can form a disulfide bond. The rate of reduction of the disulfide bond inking the therapeutic agent to the carrier is dependent on the size of the at least one functional group attached to the thiol compound.

Accordingly, it is a principal object of the present invention to provide a carrier for in vivo delivery of a therapeutic agent which can be conjugated to a carrier via a disulfide bond.

It is a further object of the invention to provide a carrier for in vivo delivery of a therapeutic agent, wherein the therapeutic agent is conjugated to the carrier via a biodegradable disulfide bond which is difficult to reduce in extracellular fluids in vivo.

It is a further object of the present invention to provide a carrier for in vivo delivery of a therapeutic agent which will remain substantially conjugated to the carrier until the carrier crosses a cell membrane. Once inside the cell, the cytosolic environment reduces the disulfide bond conjugating the therapeutic agent to the carrier, and the therapeutic agent is released to act on its target.

It is a further object of the present invention to provide a carrier for in viva delivery of a therapeutic agent which increases the bioavailability of the therapeutic agent so that the rate of repeated parenteral administration of the therapeutic agent can be minimized.

It is yet another object of the present invention to provide methods of modulating the release of a therapeutic agent in vivo wherein the release rate of the therapeutic agent from the carrier is dependent upon three dimensional configuration of a thiol compound used in methods of making carriers of the present invention.

Yet still another object of the present invention is to provide a carrier for in vivo delivery of a therapeutic agent comprising a thiol group which has a structure that can reduce the rate at which the therapeutic agent is released from the carrier. This object can be used to cause the timed release of the therapeutic agent in vivo.

It is yet another object of the present invention to provide a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the therapeutic agent is a Tat-inhibitory polypeptide derivative, analogs thereof, or biologically and pharmaceutically acceptable salts thereof.

It is yet another object of the present invention to provide a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the therapeutic agent is a Tat-inhibitory peptide derivative, and administration of a carrier of the present invention conjugated to a Tat-inhibitory peptide derivative disclosed herein can be used to treat a mammal suffering from a retroviral infection, such as AIDS.

Other objects and advantages will become apparent to those skilled in the art from a review of the ensuing description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematical drawing of Scheme I of producing a carrier for in vivo delivery of a therapeutic agent comprising a thiol group.

FIG. **2** is a schematical drawing of Scheme II for producing a carrier for in vivo delivery of a therapeutic agent comprising a thiol group.

FIG. **3** is a spectroscopic analysis of the reaction of PEG-cysteamine-thiopyridine (PEG-cysteamine-TP) with the peptide N-acetyl-Phe-Arg-Arg-Arg-Cys-NH_2 (SEQ ID NO:9). The UV spectrum of the solution of PEG-cysteamine-TP is shown (solid line). Addition of the peptide results in the release of 2-thiopyridone, which is observed as a peak with a maximum at 343 nm (dashed line).

FIG. **4** is a graph showing the purification of the product from the reaction mixture of PEG-cysteamine-TP and the peptide N-acetyl-Phe-Arg-Arg-Arg-Cys-NH_2 (SEQ ID NO:9) by gel filtration chromatography. Note the presence of the released 2-thiopyridine in the low-molecular weight fractions according to the absorbance at 343 nm.

FIG. **5** is a graph of a time course release of a radiolabeled peptide from PEG-cystemaine-peptide by 3 mM glutathione in PBS. The data is presented as a semi-log plot. Essentially the same release kinetics were obtained in two additional experiments.

FIG. **6** is a graph of a time course release of a radiolabeled peptide from PEG-1-amino-2-methyl-2-propanethiol-peptide by 30 mM glutathione in PBS. The dashed line is a theoretical plot of $C=C_{initial}e^{-kt}$ A semi-log plot of the early time point data is shown in the inset. Essentially the same release kinetics were obtained in two additional experiments.

FIG. **7** is a graph of the comparison of the inhibition of protein expression in a Jurkat cell line transfected with a TAR-CAT (TAR-Chloramphenicol Acetyl Transferase) plasmid and a Tat-protein plasmid. In this experiment, one group of cells was administered a Tat inhibitory biotinylated polypeptide derivative having a sequence of N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3), and another group of cells was administered the same Tat inhibitory biotinylated peptide derivative conjugated to a carrier of the present invention via a disulfide bond between the sulfur atom of the thiol group of the thiol compound, and the the thiol group of the Cys residue located between biotin and the amino group of the Tat inhibitory biotinylated polypeptide derivative of SEQ ID NO: 3. The polymer of the carrier used in this experiment had a molecular weight of about 27,000 D (6.29×10^4) and comprised a copolymer of polyethylene glycol (PEG) and lysine. Cysteamine was the thiol compound conjugated to the polymer. Conjugation of cysteamine to the polymer involved the carboxylic acid group of the lysine incorporated into the copolymer, which was available for reaction. If the polypeptide was successful in inhibiting Tat binding to TAR, CAT would be produced. Hence, an assay of CAT was used to measure inhibition.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on Applicants' discovery that unexpectedly, a biodegradable disulfide bond can be used to conjugate a therapeutic agent to a carrier for in vivo delivery of the therapeutic agent, wherein the carrier comprises a polymer and a thiol compound conjugated to the polymer, so that the thiol group of the thiol compound and the thiol group of the therapeutic agent can form a disulfide bond. Extracellular media in vivo does not provide an appropriate environment for appreciably reducing a disulfide bond. Hence, a disulfide bond conjugating the therapeutic agent to the polymer remains relatively stable during the circulation of the carrier throughout the intercellular matrix of the body. In contrast, cytosol of a cell provides the appropriate environment for reducing a disulfide bond, particularly since the reduced form of glutathione, a naturally occurring reducing agent, is found predominantly in the cytosol. Once the carrier crosses a cell membrane and enters the cytosol, the disulfide bond conjugating the therapeutic agent to the carrier is reduced, and the therapeutic agent is released to act upon its target in the cell. Consequently, a substantial portion of a therapeutic agent conjugated to a carrier of the present invention and administered to a subject, is delivered to target cells, and then released.

The term "about" as used herein to describe molecular weights indicates that in preparations of polymers, some molecules will weigh more, some less, than the stated molecular weight.

The term "steric hindrance" as used herein describes an effect on relative reaction rates caused by the space-filling properties of those parts of a molecule attached at or near the reacting site.

The term "bioavailability" as used herein refers to the ability of a therapeutic agent to reach its target cell or to reach its molecular target inside a cell.

The term "polymer" as used herein encompasses both homopolymers and copolymers.

As used herein, the term "interval" indicates a distance between thiol compounds conjugated to a polymer of a carrier of the present invention, or the distance between functional groups attached to a polymer of a carrier of the present invention.

Moreover, as used herein, the term "therapeutically effective" is used herein to mean an amount sufficient to prevent, and preferably reduce by at least about 30 percent, more preferably by at least 50 percent, most preferably by at least 90 percent, transactivation of viral TAR RNA by Tat protein.

The polymer of a carrier of the present invention can have either a branched or linear structure. Preferably, the polymer is a water soluble polymer.

Examples of water soluble polymers which have applications in the present invention include, but are not limited to, polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, polyaminoacids (homopolymers), polypropylene glycol, copolymers of ethylene glycol/propylene glycol, ethylene/maleic anhydride copolymer, polyaminoacids, copolymer of polyethylene glycol and an amino acid, polypropylene oxide/ethylene oxide copolymers, or polyethylene glycol/thiomalic acid copolymers. In a preferred embodiment, the polymer is a copolymer of polyethylene glycol and lysine. Methods of making such a copolymer are set forth in Nathan, A., Zalipsky, S., Ertel, S. I., Agathos, S. N., Yarmush, M. L. And Kohn, J. (1993) *Copolymers of lysine and polyethylene glycol: A new family of functionalized drug carriers.* Bioconj. Chem. 4:54–62 which is incorporated by reference herein in its entirety.

A polymer of a carrier of the present invention can have any molecular weight. In one embodiment of the present invention, the polymer has a molecular weight range of about 1,000 to about 1,000,000 Daltons, and preferably a molecular weight range of about 20,000 to 200,000 Daltons. More preferably, the polymer of a carrier of the present invention has a molecular weight of about 27,000 D.

The present invention further extends to a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer, and at least one thiol compound conjugated to the polymer at an interval, such that the thiol group of the thiol compound and the thiol group of the therapeutic agent form a disulfide bond.

In an embodiment, the interval between thiol compounds conjugated to a polymer of a carrier of the present invention is about 100 to about 10,000 Daltons. In a preferred embodiment, the interval between thiol compounds is about 300 to about 3,000 Daltons. Hence, more than one molecule of therapeutic agent can be conjugated to a carrier of the present invention, and delivered to its target tissue or cells.

Moreover, the present invention further extends to a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer comprising at least one functional group attached thereto and available for reaction, and a thiol compound conjugated to the at least one functional group, so that the thiol group of the thiol compound and the thiol group of the therapeutic agent form a disulfide bond.

Attachment of functional groups to a polymer can be accomplished with standard organic chemistry techniques. Since numerous water soluble polymers and numerous functional groups have applications in the present invention, the chemical synthetic route used to attach the at least one functional group are dependent on the polymer, and the functional group one would like to attach thereto. In a preferred embodiment of the present invention, the polymer comprises a copolymer of polyethylene glycol and lysine, which inherently has a carboxylic acid functional group attached to lysine and available for reaction.

Moreover, in an embodiment of the present invention, the functional groups are attached to the polymer at an interval of about 100 to about 10,000 Daltons. In a preferred embodiment, the interval between functional groups is about 300 to about 3,000 Daltons.

Examples of functional groups which can be attached to a polymer of a carrier of the present invention include ketones, esters, carboxylic acids, aldehydes, alcohols, thiols, or amines, to name only a few. In a preferred embodiment of the present invention, the functional group is a carboxylic acid. Moreover, more than one type of functional group can be concurrently attached to a polymer of a carrier of the present invention.

Furthermore, examples of thiol compounds having applications in a carrier of the present invention include, but are not limited to, cysteamine, 1-amino-2-methyl-2-propanethiol, or 1-amino-2-propanethiol, to name only a few.

In addition, the present invention extends to a carrier for in vivo delivery of a therapeutic agent having a thiol group, wherein the carrier comprises a polymer, a cell uptake promoter conjugated to the polymer, and at least one thiol compound conjugated to the polymer, such that the thiol group of the at least one thiol compound can form a disulfide bond with the thiol group of therapeutic agent. The cell uptake promoter enhances the ability of a carrier of the present invention conjugated to a therapeutic agent via a disulfide bond to cross a cell membrane and enter the cell's cytosol.

Numerous cell uptake promoters are known, and have applications in the present invention. An example of a cell uptake promoter having applications in the present invention is biotin. Moreover, methods of biotinylating a chemical compound, such as a carrier of the present invention, are known to the skilled artisan, and are not discussed in detail here.

Moreover, as set forth in examples described below, a cell uptake promoter can also be attached directly to the therapeutic agent comprising a thiol group, and still enhance the ability of the carrier and the therapeutic agent to cross a cell membrane.

In addition, an example of a therapeutic agent comprising a thiol group which can be conjugated to a carrier of the present invention, comprises a Tat-inhibitory binding peptide comprising a sequence of formula I:

R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-Cys-NH_2 (SEQ ID NO: 1)

wherein R is the residue of a carboxylic acid or an acetyl group, and X is a Cys or Lys residue, and analogs thereof,

and the biologically and pharmaceutically acceptable salts thereof, and stereo, optical and geometrical isomers thereof, where such isomers exist, along with pharmaceutically acceptable salts and solvates thereof. Such agents are useful in the treatment of viral infections such as HIV-1 infection in mammals, by virtue of their ability to block the interaction of Tat protein with TAR RNA, thereby interfering with the transactivation step in the replication cycle of HIV-1. The rationale for this approach is that such peptides compete with the full length Tat protein for binding to TAR RNA, thereby preventing the required interactions between other domains in Tat protein and the nascent transcription apparatus.

In another embodiment, the therapeutic agent conjugated to a carrier of the present invention is a Tat-inhibitory peptide comprising an amino acid sequence of SEQ ID NO: 8:

N-acetyl-DCys-DLys-(biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-NH_2 (SEQ ID NO:8)

along with pharmaceutically acceptable salts thereof, and analogs and derivatives thereof.

Indeed, cell culture experiments using promoter elements of the HIV-1 LTR linked to the reporter CAT gene demonstrate that therapeutic agents comprising a thiol group, as described above, and containing the 9-amino acid basic domain of Tat protein block the transactivation process.

Moreover, there is convincing evidence from a HLCE-D36 cell assay that a representative peptide of formula I, Tat10-biotin, specifically blocks Tat protein-mediated expression of CAT protein (please see Choudhury, I., Wang, J., Rabson, A. B., Stein, S., Pooyan, S., Stein, S. and Leibowitz, M. J. (1998) Inhibition of HIV-1 replication by a Tat RNA binding domain peptide analog. *J. Acqu. Immune Def. Syndr. & Human Retrovirol.*, 17, 104–111, which is incorporated by reference in its entirety). Although the mechanism whereby Tat protein induces protein expression from the HIV LTR is generally believed to be due to an effect on transcriptional elongation, it is possible that there are also post-transcriptional effects of Tat protein. Thus, whether Tat10-biotin competes with Tat protein only at the transcriptional elongation step remains to be determined. Regardless, Applicants are under no obligation to explain such a mechanism, and are not to be bound by postulates of the mechanism set forth above.

The instant invention thus provides a method of treating a retroviral infection in a mammal in need of such treatment. Such a method comprises administering to a mammal a therapeutically effective amount of a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer, and at least one thiol compound conjugated to the polymer, such that the thiol group of the thiol compound and the thiol group of the therapeutic agent form a disulfide bond, wherein the therapeutic compound comprising a thiol group comprises a Tat-inhibitory binding peptide derivative. For example, a Tat-inhibitory binding derivative having applications in this method comprises a biotinylated peptide of the formula I:

R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-Cys-NH_2 (SEQ ID NO:1)

wherein R is the residue of a carboxylic acid or an acetyl group, and X is a Cys or Lys residue, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof, or stereo, optical and geometrical isomers thereof, where such isomers exist, and pharmaceutically acceptable salts and solvates thereof.

Examples of Tat inhibitory binding derivatives of formula I include, but are not limited to:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:2)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:4)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:5)

N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:6),

or

N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:7)

or analogs or derivatives thereof, or pharmaceutically acceptable salts thereof.

In another example, a Tat-inhibitory peptide derivative which can be conjugated to a carrier of the present invention comprises an amino acid sequence of SEQ ID NO:8

N-acetyl-DCys-DLys-(biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-NH_2 (SEQ ID NO:8)

or analogs or derivatives thereof, or pharmaceutically acceptable salts thereof.

As explained above, numerous methods are readily available to synthesize such peptide derivatives. For example, they can be produced via recombinant expression of a specially engineered DNA molecule which encodes such a peptide. Moreover, such peptides can be synthesized using readily available solid phase synthesis techniques. After synthesis of such peptides, they can be readily biotinylated using presently known methods of biotinylation.

As explained above, Tat-inhibitory peptide derivatives disclosed herein can be utilized as Tat protein RNA-binding domain mimics to treat the HIV-1 infection, and the resultant AIDS. The mammal under treatment can be a human, monkey, cat or the like, with the treatment of humans being particularly preferred. A carrier for in vivo delivery, wherein the therapeutic agent comprises a Tat antagonist should also be useful for ameliorating the pathogenic effects of Tat protein on host cells due to interactions with TAR-like elements on cellular transcripts. Recent studies on peptide analogs of the core domain sequence of Tat protein, which is believed to interact with host cell factors rather than with virally encoded RNA, have lead to the same suggestion for a new class of therapeutic agents for AIDS based on inhibition of the transactivation step in the HIV-1 replication cycle. Hence, coupling such agents to a carrier of the present invention substantially reduces degradation and renal clearance of the agents in vivo prior to their interaction with the target tissue, and increases the bioavailability of such therapeutic agents to their intended target tissues and cells.

The polypeptides of formula I and their analogs, along with the amino acid sequence of SEQ ID NO:8, analogs or derivatives thereof, can be synthesized by conventional solution methods, or by solid phase synthetic techniques known in the art.

Throughout the specification and appended claims, the polypeptide of formula I, and its analogs and derivatives, and salts, encompass all stereo, optical and geometrical isomers thereof where such isomers exist, as well as the pharmaceutically acceptable salts and solvates thereof. Where appropriate, the polypeptide or its analogs can be utilized as its corresponding amide form. The amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "ID" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide.

The R group of the peptides of formula I can be any residue of an alkyl, alkenyl or aryl carboxylic acid, i.e., acetic, propionic, butyric, valeric, allylic, benzoic and the like being suitable. Particularly preferred for use in the present invention is the acetyl derivative of the peptides of formula I.

The term "biologically and pharmaceutically acceptable salts" is intended to include any such salt derived from an inorganic or organic acid which is tolerated by the mammalian system. These salts include, but are not limited to, acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, hexanoate, succinate, fumarate, hydrochloride, hydrobromide, lactate, maleate, phosphate, sulfate, methanesulfonate, oxalate, propionate, tosylate, and mesylate. Examples of acids which can be used to form such salts include such inorganic acids as hydrochloric acid, sulfuric acid and phosphoric acid, and such organic acids such as oxalic acid, maleic acid, succinic acid and citric acid.

The nomenclature used to define the polypeptides is that specified by Schroder & Lubke, "The Peptides", Academic Press (1965), wherein in accordance with conventional representation the amino group at the N-terminal appears to the left and the carboxyl group at the C-terminal to the right. NH_2 refers to the amide group present at the carboxy terminus when written at the right of a polypeptide sequence.

Accordingly, polypeptide analogs displaying substantially equivalent activity to the polypeptide of formula I are likewise contemplated for use in the present invention. These modifications can be obtained through peptide synthesis utilizing the appropriate starting material.

In keeping with standard polypeptide nomenclature, *J. Biol. Chem.*, 243:3552–59 (1969), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL		
1-Letter	3-Letter	AMINO ACID
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
C	Cys	cysteine

It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues. The above Table is presented to correlate the three-letter and one-letter notations which may appear alternately herein.

An amino acid in the polypeptide of this invention can be changed in a non-conservative manner (i.e., by changing an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting polypeptide. The present invention should be considered to include analogs whose sequences contain conservative changes which do not significantly alter the activity or binding characteristics of the resulting polypeptide.

The following is one example of various groupings of amino acids:

Amino Acids with Nonpolar R Groups
Alanine
Valine
Leucine
Isoleucine
Proline
Phenylalanine
Tryptophan
Methionine

Amino Acids with Uncharged Polar R Groups
Glycine
Serine
Threonine
Cysteine
Tyrosine
Asparagine
Glutamine

Amino Acids with Charged Polar R Groups (Negatively Charged at pH 6.0)
Aspartic acid
Glutamic acid

Basic Amino Acids (Positively Charged at pH 6.0)
Lysine
Arginine
Histidine (at pH 6.0)

Another grouping may be those amino acids with aromatic groups:
Phenylalanine
Tryptophan
Tyrosine

Another grouping may be according to molecular weight (i.e., size of R groups):

Glycine	75
Alanine	89
Serine	105
Proline	115
Valine	117
Threonine	119
Cysteine	121
Leucine	131
Isoleucine	131
Asparagine	132
Aspartic acid	133
Glutamine	146
Lysine	146
Glutamic acid	147
Methionine	149
Histidine (at pH 6.0)	155

-continued

Phenylalanine	165
Arginine	174
Tyrosine	181
Tryptophan	204

Particularly preferred substitutions are:
Gln for Arg or Lys; and
His for Lys or Arg.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys, or with a carrier of the present invention. A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces βturns in the polypeptide's structure. Alternately, D-amino acids can be substituted for the L-amino acids at one or more positions.

Representative analogs of the polypeptide of formula I thus include:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:2)
N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3)
N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:4)
N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:5)
N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:6)
N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:7)

Also disclosed herein are methods of making a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer, and at least one thiol compound conjugated to the polymer, such that the thiol group of the at least one thiol compound and the thiol group of the therapeutic agent form a disulfide bond.

One such method disclosed herein comprises the steps of:

a) reacting at least one thiol compound with a disulfide compound to form a first intermediate wherein the thiol group of the at least one thiol compound and a sulfur atom of the disulfide bond of the disulfide compound form a disulfide bond; and

b) reacting the first intermediate with the polymer to form the carrier comprising the first intermediate conjugated to the polymer.

Once the carrier has been produced, it can be reacted with a therapeutic agent comprising a thiol group, so that its disulfide bond is reduced, and the sulfur atom of the at least one thiol compound of the carrier, and the thiol group of the therapeutic agent can form a disulfide bond.

As explained above, numerous thiol compounds have applications in a method for making a carrier. Examples include cysteamine, 1-amino-2-methyl-2-propanethiol, or 1-amino-2-propanethiol, to name only a few.

Moreover, numerous disulfide compounds can be used in a method as set forth above. Preferably, the disulfide compound is symmetric. In a preferred embodiment, the compound is 2,2'-dithiodipyridine.

In addition, a polymer used in a method described above can have a branched or linear structure. Preferably, the polymer is a water soluble polymer. Examples of water soluble polymers having applications in the present invention include polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, polyaminoacids (homopolymers), polypropylene glycol, copolymers of ethylene glycol/propylene glycol, ethylene/maleic anhydride copolymer, polyaminoacids, copolymer of polyethylene glycol and an amino acid, or polypropylene oxide/ethylene oxide copolymers. In a preferred embodiment, the polymer comprises a copolymer of polyethylene glycol and lysine.

Moreover, a polymer used in methods of making a carrier of the present invention can have any molecular weight. In an embodiment of the present invention, a polymer used in a method of making a carrier for in vivo delivery of a therapeutic agent comprising a thiol group has a molecular weight range of about 1,000 to about 1,000,000 Daltons, and preferably a molecular weight range of about 20,000 to 200,000 Daltons. Preferably, the polymer comprises a molecular weight of about 27,000 Daltons.

The present invention further extends to a method of making a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the polymer comprises at least one functional group attached thereto available for reaction. Hence, a method of making such a carrier comprise the steps of:

a) reacting the thiol compound with a disulfide compound to form a first intermediate wherein the thiol group of the thiol compound and a sulfur atom of the disulfide bond of the disulfide compound form a disulfide bond; and

b) reacting the first intermediate with the polymer comprising at least one functional group attached thereto, to form the carrier comprising the first intermediate conjugated to the at least one functional group attached to the polymer.

After the carrier is formed, it can readily be reacted with a therapeutic agent comprising a thiol group so that its disulfide bond is resolved, and oxidative coupling occurs between the thiol group of the therapeutic agent, and the thiol group of the thiol compound, thereby conjugating the therapeutic agent to the carrier of the present invention via a disulfide bond.

In addition, the at least one functional group of a polymer used in a method of the present invention can be attached to the polymer at an interval. In an embodiment, the interval between functional groups on the polymer is about 100 to about 10,000 Daltons, preferably about 300 to about 3,000 Daltons.

In addition, many methods are known to the skilled artisan to react such compounds to form a first intermediate comprising a disulfide bond in a method of the present invention set forth above. For example, such a reaction can be performed in the presence of an oxidizing agent, such as oxygen, O_2, hydrogen peroxide, thallium(III) acetate, Me_2SOI_2, bromine under phase transfer conditions, nitric oxide, potassium dichromate, copper, pyridinium chlorochromate (PCC) when dissolved in methylene chloride, to name only a few.

After the first intermediate is formed in the method described above, numerous chemical reactions are available to conjugate the first intermediate to the at least one functional group of the polymer without disrupting the disulfide bond of the first intermediate. For example, such a reaction can be carried out in a polar aprotic solvent, such as N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), dimethylacetamide (DMA). Preferably, the reaction is carried out in a solution comprising DMF, and other reagents such as benzotriazol-1-yl-oxytris(dimethylamino)-

phosphonium hexafluorophosphate (BOP) and hydroxybenzotriazol (HOBt). Other reactions apparent to the skilled artisan which conjugate the first intermediate to at least one functional group of a polymer of a carrier of the present invention are encompassed by the present invention.

After the carrier of the present invention is formed, it can be reacted with a therapeutic agent comprising a thiol group, so that the sulfur atom of the thiol compound and the thiol group of the therapeutic agent can form a disulfide bond. This reaction involves reducing the disulfide bond of the carrier so that the sulfur atom of the thiol compound conjugated to the at least one functional group of the polymer undergoes an oxidative coupling with the thiol group of the therapeutic agent. As a result, the therapeutic agent is coupled to a carrier of the present invention via a biodegradable disulfide bond.

Another method of making a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, as set forth herein, comprises the steps of:

a) reacting a first thiol compound with a second thiol compound to form a first intermediate wherein the thiol group of the first thiol compound and the thiol group of the second thiol compound form a disulfide bond;

b) reacting the first intermediate with a polymer, to form a second intermediate comprising the first intermediate conjugated to the polymer;

c) reducing the disulfide bond of the second intermediate to form a third intermediate comprising the polymer and the first thiol compound conjugated to the polymer so that the thiol group of the first thiol compound is available for reaction; and

d) reacting the third intermediate with a disulfide compound to form the carrier, wherein the thiol group of the first thiol compound and a sulfur atom of the disulfide compound form a disulfide bond.

After the carrier is formed, it can readily be reacted with a therapeutic agent comprising a thiol group. Such a reaction involves reducing the disulfide bond of the carrier, and promoting oxidative coupling between the sulfur atom of the first thiol compound and the thiol group of the therapeutic agent to form a biodegradable disulfide bond.

In an embodiment of the present invention, the first thiol compound and the second thiol compound are the same compound. Hence, the first intermediate can be either a symmetric or asymmetric disulfide compound. Such a reaction can be readily carried out using oxidizing agents set forth above.

Moreover, the conjugation of the first intermediate to the polymer of a carrier of the present invention to form the second intermediate can be carried out in a manner similar to that described above, i.e. in a aprotic polar solvent, with the addition of BOP and HOBt.

The next step of this method of the present invention involves reduction of the disulfide bond of the second intermediate to form the third intermediate. Numerous reducing agents are available to the skilled artisan to reduce the disulfide bond. Examples include lithium aluminum hydride ($LiAlH_4$) in the presence of diethylether, sodium borohydride ($NaBH_4$), glutathione, β-mercaptoethanol, and dithiothrietol (DTT), to name only a few.

Once the thiol group is formed on the third intermediate, it can be reacted with a disulfide compound, in order to form a carrier of the present invention. Preferably, the disulfide compound is symmetric. In an embodiment of the present invention, the compound comprising a disulfide bond is 2,2'-dithiodipyridine. In this embodiment, the disulfide bond of this compound can easily be reduced to form two thiopyridine molecules, one which acts as a leaving group, and one which undergoes oxidative coupling with the thiol group of the third intermediate to form a carrier of the present invention.

An additional step can involve reacting the carrier with a therapeutic compound comprising a thiol group. Such a reaction readily occurs, since the thiopyridine is a stable leaving group. As a result, the therapeutic agent can be conjugated to a carrier of the present invention via a biodegradable disulfide bond, which is difficult to reduce in extracellular fluids in vivo, but is reduced in the cytosol. Hence, a carrier of the present invention provides a therapeutic agent with increased bioavailability relative to bioavailability provided to therapeutic agents with other types of carriers discussed above.

In another embodiment of the present invention, at least one functional group is attached to a polymer of a carrier of the present invention, and is available for reaction. A method of producing a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, comprises the steps of:

a) reacting a first thiol compound with a second thiol compound to form a first intermediate wherein the thiol group of the first thiol compound and the thiol group of the second thiol compound form a disulfide bond;

b) reacting the first intermediate with a polymer comprising at least one functional group attached thereto, to form a second intermediate comprising the first intermediate conjugated to the at least one functional group;

c) reducing the disulfide bond of the second intermediate to form a third intermediate comprising the polymer comprising at least one functional group attached thereto, and the first thiol compound conjugated to the functional group, so that the thiol group of the first thiol compound is available for reaction; and

d) reacting the third intermediate with a disulfide compound to form the carrier, wherein the thiol group of the first thiol compound and a sulfur atom of the disulfide compound form a disulfide bond.

As explained above, numerous functional groups have applications in a method described above. Examples of functional groups which can be attached to a polymer used of a carrier of the present invention include ketones, esters, carboxylic acids, aldehydes, alcohols, thiols, or amines, to name only a few. In a preferred embodiment of the present invention, the functional group is a carboxylic acid. Moreover, more than one type of functional group can be concurrently attached to a polymer of a carrier of the present invention.

In addition, the least one functional group can be attached to a polymer at an interval. In an embodiment, the interval between functional groups on the polymer is about 100 to about 10,000 Daltons. In a preferred embodiment, the interval between functional groups on the polymer is about 300 to about 3,000 Daltons.

Moreover, numerous thiol compounds have applications in methods of the present invention. For example, thiol compounds such as cysteamine, 1-amino-2-methyl-2-propanethiol, or 1-amino-2-propanethiol can serve as the thiol compound of this method of the present invention, to name only a few.

Further, just as for other methods described above for making a carrier of the present invention, the polymer in this method of the present invention can have a branched or linear structure. Preferably, the polymer is a water soluble polymer. Examples of water soluble polymers having applications herein include polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvi-

nyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, polyaminoacids (homopolymers), polypropylene glycol, copolymers of ethylene glycol/propylene glycol, ethylene/maleic anhydride copolymer, polyaminoacids, copolymer of polyethylene glycol and an amino acid, polypropylene oxide/ethylene oxide copolymers, or polyethylene glycol/thiomalic acid copolymers. In a preferred embodiment, the polymer comprises a copolymer of polyethylene glycol and lysine, which has a carboxylic acid functional group readily available to conjugate a thiol compound to a polymer of a carrier of the present invention.

In addition, a polymer can have any molecular weight. In an embodiment of the present invention, the polymer has a molecular weight range of about 1,000 to about 1,000,000 Daltons, and preferably a molecular weight range of about 20,000 to 200,000 Daltons. In a preferred embodiment, the polymer is a copolymer of polyethylene glycol and lysine, and has a molecular weight of about 27,000 Daltons.

Examples of therapeutic agents comprising a thiol group are also set forth throughout the Specification. However, the present invention and its uses are by no means limited to the examples set forth herein. In particular, disclosed herein are Tat-inhibitory binding peptide derivatives which comprise a thiol group. Hence, they can readily be conjugated to a carrier of the present invention via disulfide bond, and administered in therapeutically effective amounts to treat a mammal suffering from a viral infection, such as AIDS. An example of a Tat-inhibitory peptide derivative having applications in the present invention comprises an amino acid sequence of formula I:

R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-Cys-NH_2 (SEQ ID NO:1)

wherein R is the residue of a carboxylic acid or acetyl group, and X is a Cys or Lys residue, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof, and stereo, optical and geometrical isomers thereof, where such isomers exist, and pharmaceutically acceptable salts and solvates thereof. Since such agents include a Cys residue, which comprises a thiol group, they can be readily conjugated via a disulfide bond to a carrier of the present invention.

Representative analogs of the polypeptide of formula I thus include:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:2)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:4)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:5)

N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:6)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:7)

In another example of a therapeutic agent comprising a thiol group which can be conjugated to a carrier of the present invention, disclosed herein is a Tat-inhibitory binding peptide comprising an amino acid sequence as set forth in SEQ ID NO: 8:

N-acetyl-DCys-DLys-(biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-NH_2 (SEQ ID NO:8)

along with analogs or derivatives thereof, and biologically and pharmaceutically acceptable salts thereof.

Furthermore, as explained above, the present invention extends to a method of treating a viral infection in a mammal in need of such treatment. More particularly, a such a method comprises administering to a mammal a therapeutically effective amount of a therapeutic agent comprising a thiol group, which is conjugated to a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer, and at least one thiol compound conjugated to the polymer, such that the thiol group of the thiol compound and the thiol group of the therapeutic agent form a disulfide bond, wherein the therapeutic agent comprises a Tat-inhibitory binding peptide derivative.

Examples of a Tat-inhibitory binding peptide derivative comprises a biotinylated peptide of the formula I:

R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-Cys-NH_2 (SEQ ID NO: 1)

wherein R is the residue of a carboxylic acid or acetyl group, and X is a Cys or Lys residue, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof, and stereo, optical and geometrical isomers thereof, where such isomers exist.

Moreover, an additional examples of a Tat-inhibitory binding peptide derivative which can be conjugated to a carrier of the present invention, and administered in therapeutically effective amounts to a mammal suffering from a viral infection, such as AIDS, include, but are not limited to:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:2)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:4)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:5)

N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:6), or

N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys (biotin)-Cys-NH_2 (SEQ ID NO:7) to name only a few.

Another example of a therapeutic agent comprising a thiol group which is conjugated to a carrier of the present invention and can be used in treating a viral infection in a mammal in need of such treatment, comprises an amino acid sequence set forth in SEQ ID NO:8:

N-acetyl-DCys-DLys-(biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-NH_2 (SEQ ID NO:8)

along with analogs or derivatives thereof and pharmaceutically acceptable salts thereof.

In particular, the method can be used to treat retroviral infections such as AIDS in humans.

Numerous methods are presently available for administering the carrier of the present invention to a mammal, which will produce a therapeutic effect therein. For example, a pharmaceutical composition can be formed comprising a carrier of the present invention conjugated to a Tat-inhibitory peptide derivative, and a pharmaceutically acceptable carrier thereof. Moreover, according to the invention, the carrier conjugated to a Tat-inhibitory peptide derivative, or a pharmaceutical composition as described above, may be introduced parenterally, transmucosally, e.g., orally, nasally, or rectally, or transdermally. Preferably, administration is parenteral, e.g., via intravenous injection, and also includes, but is not limited to, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial administration.

In another embodiment, the carrier of the present invention is conjugated to a Tat-protein inhibitory polypeptide derivative, or pharmaceutical composition comprising such a conjugated species, can be delivered in a vesicle, in particular a liposome [see Langer, *Science* 249:1527–1533

(1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss: New York, pp. 353–365 (1989); Lopez-Berestein, ibid., pp. 317–327; see generally ibid.].

In yet another embodiment, the therapeutic agent, such as a Tat-protein inhibitory polypeptide derivative can be conjugated to a carrier of the present invention, which can then be delivered in a controlled release system. For example, as explained above, functional groups attached to a thiol group of a carrier of the present invention produce steric hindrance which reduces the rate at which a disulfide bond conjugating the therapeutic agent to a carrier of the present invention is reduced. Hence the rate at which a Tat-protein inhibitory polypeptide derivative is released from the carrier, and delivered to a target cell or tissue can be modulated, depending on the structure of the thiol compound of the carrier. Other ways of delivering the therapeutic agent in a controlled release system include administration via intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used [see Langer, supra; Sefton, *CRC Ctit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)]. In another embodiment, polymeric materials can be used [see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Press: Boca Raton, Fla. (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley: New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); see also Levy et al., *Science* 228:190 (1985); During et al., *Ann. Neurol.* 25:351 (1989); Howard et al., *J. Neurosurg.* 71:105 (1989)].

The following example is presented in order to more fully illustrate the invention. It should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE

As explained above, covalent attachment of water-soluble polymers can be used to manipulate pharmacologic properties of a therapeutic agent. For example, appending polyethylene glycol (PEG) chains to a protein can increase its circulating half-life and minimize its immunologic properties[2]. Other researchers have designed a PEG-lysine copolymer having multiple attachment sites[3]. The researchers then prepared conjugates through either biostable or biodegradable linkages[3], but did not investigate the biodegradable disulfide linkage. This linkage can be especially useful for drug delivery into cells, due to the stronger reducing environment within cells than in extracellular fluids. Disclosed herein is a carrier for in vivo delivery of a therapeutic agent comprising a thiol group, in which the carrier has multiple attachment sites for forming a disulfide bond with a therapeutic agent. Disulfide bonds have heretofore not been used as a biodegradable linkage between a therapeutic agent and a synthetic polymer carrier, nor in combination with a cell uptake promoter.

Materials and Methods

Cysteamine hydrochloride, Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid) and glutathione (reduced form) were obtained from Sigma Chemicals (St. Louis, Mo.). Dithiothreitol (DTT) was obtained from Pierce (Rockford Ill.). 2,2'-dipyridyl disulfate, 1-amino2-methyl-2-propanethiol, diisopropylethyl amine (DIEA) and silica gel, 70–230 mesh, 60A were obtained from Aldrich Chemical (Milwaukee, Wis.). Hydroxybenzotriazol (HOBt), benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and dimethylformamide (DMF) were obtained from PerSeptive Biosystems (Boston, Mass.). Dichloromethane (DCM) and methanol (MeOH) were HPLC grade and obtained from Fisher Scientific (Pittsburgh, Pa.). "SEPHADEX" gel filtration beads were obtained from Pharmacia LKB Biotechnology (Piscataway, N.J.). Polyethylene glycol-lysine copolymer with a molecular weight of about 27 kDa, (Molecular Weight=2.69×10^4 D, 2198 D/repeating unit), was synthesized according to a published procedure[3].

Peptide Synthesis

Small peptides were synthesized and used herein as therapeutic agents comprising thiol groups. The peptide N-acetyl-Phe-Arg-Arg-Arg-Cys-NH_2 (SEQ ID NO:9) was synthesized at Louisiana State University (LSU) core facility using Fmoc chemistry, purified by reverse-phase HPLC and confirmed for structure by mass spectral analysis.

The peptides N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-NH_2 (SEQ ID NO:10), and N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3) were manually synthesized using Fmoc chemistry. For radiolabeling, the assembled peptide on the solid support was allowed to react with tritiated acetic anhydride in the presence of the coupling activation reagents, BOP, HOBt, and DIEA. Acetylation of the peptide was completed by chasing with an excess of non-radioactive acetic anhydride.

After cleavage from the solid support followed by ether precipitation, the radiolabeled peptide was purified by chromatography on "SEPHADEX" gel filtration beads size G-10 using PBS (0.15 M NaCl, 20 mM potassium phosphate buffer, pH 7.4) for elution or by centrifugal concentration using a "CENTRICON-10" ultrafilter (Amicon/Millipore, Bedford, Mass.).

Preparation of a Carrier of the Present Invention with Scheme I

To a solution of cysteamine hydrogen chloride (0.50 g, 4.4 mmole) dissolved in 15 ml degassed buffer (0.1 N sodium acetate adjusted to pH 4.0 with acetic acid, 0.3 M sodium chloride, 1 mM EDTA) was added 2,2'-dithiodipyridine (3.5 g, 15 mmol; dissolved in 18 ml MeOH). The mixture was stirred at room temperature under N_2. The reaction, which was monitored with thin layer chromatography (ammonium hydroxide/MeOH (methanol)/DCM (dichloromethane): Feb. 10, 1990), was complete after 4 h. The MeOH was removed in vacuo and the residue was made basic by saturating with sodium carbonate. The residue was extracted into DCM (3×50 ml) and the combined DCM was washed with water (2×40 ml) and brine (40 ml) and then dried over magnesium sulfate. The magnesium sulfate was filtered and the filtrate was concentrated in vacuo to an oily residue. The product, cysteamine-thiopyridine (the first intermediate of scheme I), was purified by flash chromatography on a silica gel column (ammonium hydroxide/MeOH/DCM: Feb. 10, 1990). Yield: 0.30 g (yellow oil) (36%). ^{1}HNMR (CDC_{13}) δ 8.4–8.5 ppm (m,1H), 7.7–7.6 ppm (m,2H), 7.15–7.0 ppm (m,1H), 3.0–2.7 ppm (m, 4H), 0.9 ppm (b, 2H).

The polymer comprising PEG-lysine copolymer (0.20 g, 91 μmol lysine) was dissolved in 2.5 ml of degassed DMF. Cysteamine-TP (the first intermediate) (0.5 mmol), BOP (0.5 mmol), HOBt (0.5 mmol) and DIEA (25 μl) were added to the solution comprising the polymer, and stirred under N_2 overnight. In this reaction, the first intermediate conjugates

with the at least one carboxylic acid available for reaction attached to the polymer to form the second intermediate. Since numerous free carboxylic acids are present on the polymer, more than one first intermediate can be conjugated to the polymer.

The product (a carrier of the present invention) was precipitated with cold ether, washed with cold ether and then dried by Speedvac (Savant Instrument Co., Farmingdale, N.Y.) to a gel-like residue. The product was dissolved, and purified on a "SEPHADEX" gel filtration G-75 column (48 ml bed volume) using 0.1 N acetic acid. Appropriate fractions were combined and lyophilized. Yield: 0.11 g (colorless solid). Once the carrier has been purified, it can be reacted with a therapeutic agent comprising a thiol group so that the disulfide bond of the carrier is reduced, and a new disulfide bond can be formed conjugating the therapeutic agent to the carrier.

Preparation of a Carrier of the Present Invention with Scheme II

As stated above, in this method, the first thiol compound and the second thiol compound can be the same chemical compound. Hence, in this example, two cysteamine hydrogen chlorides were first converted to a disulfide-linked dimer by overnight stirring of an in air aqueous solution (0.3 g in 15 ml) adjusted to pH 8.6 with triethylamine. Ellman's assay indicated less than 1% remaining-free thiol. To the mixture was added a saturated solution of sodium carbonate to pH 10, followed by extracting into DCM (5×20 ml), drying over magnesium sulfate, filtering and concentrating on a rotary evaporator. As a result, a first intermediate was formed which is a symmetric disulfide.

PEG-lysine copolymer (0.20 g, 91 micromol of lysine, molecular weight 2.69×10^4 D) was dissolved in 2.5 ml of degassed DMF. The cysteamine dimer (the first intermediate) (650 μmole), BOP (650 μmole), HOBt (650 μmole) and DIEA (25 μl) were added to the PEG copolymer solution and stirred under N_2 overnight. The product was precipitated with cold ether, washed with cold ether and then dried by Speedvac to a gel. The product, the second intermediate, was purified on a "SEPHADEX" G-75 gel filtration column, as above. Appropriate fractions were combined and lyophilized to give 70 mg of colorless solid, which was dissolved in degassed PBS (pH 7.4).

DTT (10 molar equivalents based on lysine) was then added to reduce the disulfide bond of the second intermediate, and the reduction reaction proceeded under N_2 overnight. The product, the third intermediate, was purified on a G-75 column (48 ml bed volume) using 0.1 N acetic acid containing 1 mM EDTA for elution. Appropriate fractions were combined and lyophilized to give 60 mg of colorless solid, which was dissolved in 4 ml of degassed buffer (0.1 N sodium acetate adjusted to pH 4 with acetic acid, 0.3 M sodium chloride, 1 mM EDTA).

2,2'Dithiopyridine (270 μmole) dissolved in 3 ml of degassed MeOH was added and the reaction mixture was stirred under N_2 for 2 h. The MeOH was removed on a rotary evaporator and the product (the carrier) was purified on a "SEPHADEX" G-75 gel filtration column (bed volume 48 ml) using 0.1 N acetic acid for elution. Appropriate fractions were combined, and lyophilized to give 30 mg of colorless solid. The carrier, which at this point is an asymmetric disulfide, can readily be reacted with a therapeutic agent comprising a thiol group so that its disulfide bond can be reduced, and the therapeutic agent can be conjugated to the carrier of the present invention via a disulfide bond. A similar procedure was used to prepare the PEG derivative of the sterically hindered cysteamine analog, 1-amino-2-methyl-2-propanethiol. The use of the sterically hindered cysteamine analog in a carrier of the present invention can modulate the rate of release of a therapeutic agent from the carrier. In particular, the addition of the 2 methyl groups to cysteamine causes steric hindrance. Applicants have discovered this hindrance reduces the rate at which the disulfide bond conjugating a therapeutic agent to a carrier of the invention is reduced. As a result, the rate at which the therapeutic agent is released from the carrier is reduced. This reduction of rate of release is dependent upon the steric hindrance of the thiol compound conjugated to the polymer of a carrier of the present invention.

Determination of the 2-Thiopyridine Content in PEG-S-S-TP.

The concentration of a PEG-S-S-TP solution was determined by acid hydrolysis and measurement of the released lysine using an Applied Biosystems amino acid analyzer (LSU core facility). Varying amounts of PEG-S-S-2TP (100, 200 and 400 μl) of a 4 mg/ml (ca. 2 mol/ml of lysine) solution in water were reacted separately with an excess of DTT (200 μl of 26 mole/ml in water) and diluted to a final volume of 1.0 ml with PBS. The amount of 2-thiopyridone liberated was quantitated using a molar extinction coefficient of 7.06×103 at 343 nm (5). The ratio of Thiopyridine to lysine was then calculated.

Preparation of PEG-Cysteamine-Peptide.

PEG-cysteamine-TP (an example of a carrier of the present invention prior to its conjugation via a disulfide bond to a therapeutic agent comprising a thiol group), dissolved in water (8 μmole/ml of lysine), was mixed with an equal volume of PBS containing 1 mM EDTA. To 3 ml of this solution was added 1 equivalent (as determined by Ellman's assay for thiolse) of the peptide, N-acetyl-Phe-Arg-Arg-Arg-Cys-NH_2 (SEQ ID NO:9). The reaction, which was monitored at 343 nm on aliquots of the mixture, was complete after 15 minutes of stirring under N_2. The product was purified on a Sephadex G-75 column, using PBS as eluent. Appropriate fractions were pooled. The extent of peptide derivatization of the copolymer was determined by amino acid analysis (LSU core facility). Radiolabeled peptides were appended to PEG-cysteamine-TP and purified on a G-75 column in a similar manner, but on a smaller scale.

Release of Peptides from a Carrier of the Present Invention

The disulfide linked conjugate, PEG-cysteamine-N-acetyl-Phe-Arg-Arg-Arg-Cys-NH_2 (SEQ ID NO:9) was added to a solution of reduced glutathione (3 mM in PBS) to a final peptide concentration of 0.3 mM, and then incubated under N_2 at room temperature. A sample was taken at each time point, acidified by adding an aliquot of 5% TFA and applied to a G-75 column in 0. 1% TFA (7 ml bed volume) to separate PEG-conjugated peptide from free peptide. Appropriate fractions in the high-molecular-weight region were pooled, and the extent of peptide derivatization at each time point was determined by amino acid analysis. For measuring the release of radiolabeled peptide, each time point aliquot was acidified to pH 3.5 with sulfuric acid. The PEG-conjugated peptide and the free peptide were then separated using ultrafiltration on "CENTRICON-10". To ensure complete separation, the retentate was diluted and ultrafiltered two more times. Radioactivity in the retentate and combined filtrate was quantitated by scintillation counting.

RESULTS

Preparation of a Carrier of the Present Invention Conjugated to a Therapeutic Agent Comprising a Thiol Group

The synthetic routes are given in Schemes 1 and II. In Scheme 1, cysteamine, the thiol compound, was first converted into the mixed disulfide with 2-thiopyridine, forming the first intermediate. The cysteamine-TP reagent was purified by silica gel chromatography and its structure was confirmed by ^{1}H-NMR. It was found to be necessary to use this reagent immediately for coupling to the PEG-lysine copolymer, since the 2-thiopyridone would react back to 2,2-dipyridyldisulfide, as evaluated by NMR, at a rate of about 15%/day. Amide bond formation between the carboxylate groups on the PEG-lysine copolymer and the amino group on cysteamine was accomplished using reagents typically used for coupling in peptide synthesis (Scheme I).

In Scheme II, cysteamine (the first and second thiol compounds in the example) is first converted to the symmetric disulfide (the first intermediate of this method), then appended to the PEG-lysine polymer forming the second intermediate. The disulfide bond of the second intermediate is then reduced to form the third intermediate comprising a thiol group. In the last step, the third derivative is reacted with 2,2'-dithiodipyridine to produce a carrier of the present invention prior to its conjugation to a therapeutic agent comprising a thiol group. Hence at this point, the carrier comprises a disulfide bond.

The disulfide bond between 2-thiopyridine and cysteamine appended to PEG-lysine polymer of the carrier is stable to hydrolysis in PBS (pH 7.4), as determined by absence of absorbance at 343 nm after 24 hours at room temperature. However, upon addition of a cysteinyl-peptide, such as N-acetyl-Phe-Arg-Arg-Arg-Cys-NH_2 (SEQ ID NO:9), the reaction was complete within several minutes, as determined by the released 2-thiopyridone group, measured at 343 nm (FIG. 3). With regard to stoichiometry, the amount of 2-thiopyridone released from a given amount of PEG/Lys-S-S-TP solution by an excess of reducing agent (DTT) in PBS was determined. One equivalent (as defined by thiol groups measured by Ellman's assay) of the peptide, N-acetyl-Phe-Arg-Arg-Arg-Cys-NH_2 (SEQ ID NO:9), was then added to an aliquot of PEG/Lys-S-S-TP solution, and the reaction was found to be essentially complete according to released 2-thiopyridone. Purification of the product, PEG/Lys-S-S-PEPTIDE, by gel filtration chromatography gave complete separation from released 2-thiopyridone and, presumably, from any residual unreacted peptide (FIG. 4).

Acid hydrolysis, followed by amino acid analysis was used to determine the concentration of a sample of the PEG-lysine copolymer, and treatment with an excess of i)TT was used to determine the amount of coupled cysteamine-S-S-TP on that same sample. By this procedure, it was determined that 66+/−8% of the lysine carboxylate groups had been derivatized with cysteamine-TP on a sample of PEG/Lys cysteamine-TP prepared by Scheme I. Acid hydrolysis, followed by amino acid analysis was also used to determine the extent of derivatization by the peptide, N-acetyl-Phe-Arg-Arg-Arg-Cys-NH_2 (SEQ ID NO:9). Duplicate analyses gave a ratio of Phe:Lys of 0.64 and 0.55, and of Arg:Lys of 2.0 and 1.8. Therefore, the extent of peptide derivatization, based on lysine groups, was 62+/−4%. In another sample prepared by Scheme I, the peptide coupling ratio was found to be 66+/−31%. In a sample prepared by Scheme II, the peptide coupling ratio was found to be78+/−9%.

Release Studies

Release of the peptide, N-acetyl-Phe-Arg-Arg-Arg-Cys-NH_2 (SEQ ID NO:9), by reductive cleavage of its disulfide linkage to a carrier of the present invention comprising a PEG/Lys copolymer was evaluated using 3 mM glutathione in PBS (pH 7.4) at room temperature. At each time point, an aliquot was withdrawn and acidified to halt the disulfide interchange reaction. Released peptide was removed from PEG/Lys-peptide conjugate by gel filtration chromatography, and the ratio of PEG (i.e. Lys residues) to peptide (i.e. Phe or Arg residues) was determined by amino acid analysis. Release was found to be relatively rapid, with a half-time of about 3 minutes. The presence of glycine and glutamic acid in the high-molecular-weight glutathione-treated samples (data not shown) indicates that glutathione replaces the released peptide on the PEG copolymer.

Release kinetics were also studied using the disulfide-linked radiolabeled peptides, N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-NH_2 (SEQ ID NO: 10) and N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3). Released peptide was removed from PEG/Lys-peptide conjugate by ultrafiltration. The half time of release in PBS containing 3 mM glutathione was found to also be about 3 minutes for either peptide (FIG. 5).

To achieve a slower release rate, as previously suggested for linking antibodies to toxins[7], the PEG/Lys derivative of the sterically hindered cysteamine analog, 1-amino-2-methyl-2-propanethiol, was synthesized. Analysis of the release rate of the radiolabeled peptide, N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3) gave a half-time of about 40 min in the presence of 30 mM glutathione (FIG. 6). Since the disulfide cleavage rate is proportional to glutathione concentrations, the sterically hindered linker has a release rate about 100 times slower than does a carrier of the present invention in which the thiol compound is cysteamine. Thus, the release rate can be readily controlled by selection of a thiol compound having functional groups, wherein the thiol compound is conjugated to the polymer, and conjugated via a disulfide bond to a therapeutic agent.

Bioavailability of Therapeutic Agents Conjugated to a Carrier of the Present Invention

In order to determine the effect of a carrier of the present invention on bioavailability of a therapeutic agent conjugated thereto, a Tat-inhibitory polypeptide derivative, having an amino acid sequence of N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3) was conjugated to a carrier of the present invention via a disulfide bond through an eleventh residue of cysteine. The carrier of the present invention used in this example comprised a polymer comprising a polyethylene glycol/lysine copolymer with a molecular weight of about 27,000 D (2.69×10^4 D). The at least one thiol compound conjugated to the polymer was cysteamine. In particular, cysteamine was conjugated to the carboxylic acid group of lysine of the polymer (hereinafter referred to as the "cysteamine-PEG carrier").

An experiment was then performed to compare the potency of the amino acid sequence of SEQ ID NO:3 alone to inhibit Tat binding to TAR RNA with the potency of the amino acid sequence of SEQ ID NO:3 conjugated to a

carrier of the present invention to inhibit Tat binding to TAR RNA. The results of this experiment are set forth in FIG. 7.

Initially, a Jurkat cell line was stably transfected with a TAR-CAT plasmid. At time (t)=0, the cells were transfected with a Tat-protein plasmid. The cells were then grown in 10 % fetal calf serum for about 18 hours. At t=18 hours, the biotinylated Tat peptide inhibitor, having a sequence of SEQ ID NO:3, either free or appended to a carrier of the present invention via disulfide bond, wherein the carrier comprised a PEG/Lys copolymer having a molecular weight of about 27,000 D (2.69×10^4 D) with cysteamine conjugated to the polymer, and a disulfide bond formed between the inhibitor having an amino acid of SEQ ID NO:3 and cysteamine, was added at each indicated concentration set forth in x axis of the graph of FIG. 7. At t=42 hours, the cells were harvested and CAT protein was measured by immunoassay. Each data point set forth in FIG. 7 is the average of 3 separate cell cultures. The data for the low concentrations of free and appended peptide were obtained in a side-by-side experiment, whereas the data for the high concentrations were obtained in separate experiments. The remaining 20–25% of CAT activity at high inhibitor concentrations most likely represents CAT protein already synthesized prior to addition of the inhibitor to the culture media and its uptake by the cells.

Surprisingly, the biotinylated Tat-peptide inhibitor (comprising an amino acid sequence of SEQ ID NO:3) appended to a carrier of the present invention had an additional about 5-fold increase in potency relative to the potency of the biotinylated Tat-peptide inhibitor administered alone. Hence, a carrier of the present invention contributes to enhanced potency and bioavailability of a therapeutic agent comprising a thiol group.

DISCUSSION

Disclosed herein are carriers for in vivo delivery of a therapeutic agent, wherein the therapeutic agent is reversibly linked to a carrier of the present invention, and methods for making such carriers. The reversible linkage is a disulfide bond, which can be cleaved by glutathione, a physiologically relevant reducing agent found predominantly in cellular cytosol.

In particular, release of a therapeutic agent, such as a peptide, from a carrier of the present invention was studied in PBS containing 3 mM reduced glutathione, which is the approximate concentration found in the cells of most tissues[9]. Since the concentration of reduced glutathione in blood is on the order of 10 μM[10], a therapeutic agent conjugated to a carrier of the present invention via a disulfide bond should remain mostly bound to a carrier of the present invention in an extracellular environment. More importantly, therapeutic agents should be released from a carrier of the present invention upon entry into cells.

Moreover, the peptide, N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3), used in the examples disclosed herein, represents a Tat-inhibitory peptide derivative, as set forth above. The appended biotin moiety has been found to increase cell uptake of the peptide[11]. Such a cell uptake promoter can be appended either to the therapeutic agent, or to the polymer of a carrier of the present invention. Hence, a carrier of the present invention provides a vehicle for reaching intracellular disease-related targets with therapeutic agents that would otherwise not have the requisite pharmacological properties to cross a cell membrane. The appended Cys serves as the thiol group of the therapeutic agent for forming the disulfide bond with the carrier.

In addition, data set forth in FIG. 7 clearly indicates a therapeutic agent has increased potency when conjugated to a carrier of the present invention. Such increased potency is a reflection of protection and increased bioavailability the carrier of the present invention provides to a therapeutic agent conjugated to it.

While the invention has been described and illustrated herein by reference to various specific materials, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material, and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art.

The following is a list of documents related to the above disclosure and particularly to the experimental procedures and discussions. The documents should be considered as incorporated by reference in their entirety.

1. Duncan, R. and Kopacek, J. (1984) Soluble synthetic polymers as potential drug carriers. *Adv. Polym. Sci.* 57, 53–101.
2. Davis, S., Abuchowski, A., Park, Y. K. and Davis, F. F. (1981) Alteration of the circulating half life and antigenic properties of bovine adenosine deaminase in mice by attachment of polyethylene glycol. *Clin. Exp. Immunol.* 46, 649–652.
3. Nathan, A., Zalipsky, S., Ertel, S. I., Agathos, S. N., Yarmush, M. L. and Kohn, J. (1993) Copolymers of lysine and polyethylene glycol: A new family of functionalized drug carriers. *Bioconj. Chem.* 4, 54–62.
4. Woghiren, C., Sharma, B. and Stein, S. (1993) Protected thiolpolyethylene glycol: A new activated polymer for reversible protein modification. *Bioconj. Chem.* 4, 314–318.
5. D. R. Grassetti and J. F. Murray. J. R. (1967) Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiodipyridine. Arch. Biochem. Biophys. 119, 41–49.
6. Riddles, P.W., Blakeley, R.L., and Zerner, B. (1979) Ellman's reagent:5,5'Dithiobis(2-nitrobenzoic acid)-a reexamination. *Anal. Biochem.* 94, 75–81.
7. Goff, D. A. and Carroll, S. F. (1990) Substituted 2-iminothiolanes: Reagents for the preparation of disulfide cross-linked conjugates with increased stability. *Bioconj. Chem.* 1, 381–386.
8. Trimble, S. P., Marquardt, D. and Anderson, D. C. (1997) Use of designed peptide linkers and recombinant hemoglobin mutants for drug delivery: In vitro release of an angiotensin II analog and kinetic modeling of delivery. *Bioconj. Chem.* 8, 416–423.
9. Meister, A., Griffith, 0. W. and-Tate, S. S. (1979) New aspects of glutathione metabolism and translocation in mammals. *Ciba Foundation Symposium* 72, 135–161.
10. Meister, A. (1991) Glutathione deficiency produced by inhibition of its synthesis and its reversal; applications in research and therapy. *Pharmacol. Ther.* 51, 155–194.
11. Choudhury, I., Wang, J., Rabson, A. B., Stein, S., Pooyan, S., Stein, S. and Leibowitz, M. J. (1998) Inhibition of HIV-1 replication by a Tat RNA binding domain peptide analog. *J. Acqu. Immune Def. Syndr. & Human Retrovirol.*, 17, 104–111 .

Moreover, any previously cited references are also incorporated by reference in their entirety.

Many other variations and modifications of the invention will be apparent to those skilled in the art without departing from the scope of the invention. The above-described descriptions are, therefore, intended to be merely exemplary, and all such variations and modifications are intended to be included within the scope of the invention as defined in the

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Arg Lys Lys Arg Arg Gln Arg Arg Arg Lys Cys
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<400> SEQUENCE: 9

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1 5

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<400> SEQUENCE: 10

Arg Lys Lys Arg Arg Gln Arg Arg Arg Cys
1 5 10

What is claimed is:

1. A carrier for in-vivo delivery of a therapeutic agent comprising a thiol group, wherein said carrier comprises:

a) a polymer;

b) multiple thiol compounds each comprising a sulfur atom conjugated to said polymer such that said sulfur atom of said thiol compound and said thiol group of said therapeutic agent can form a disulfide bond; and

c) a cell uptake promoter conjugated to said polymer.

2. The carrier of claim 1, wherein said polymer comprises a branched or linear structure.

3. The carrier of claim 1, wherein said polymer is a water soluble polymer.

4. The carrier of claim 3, wherein said water soluble polymer is selected from the group consisting of polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, amino acid homopolymers, polypropylene glycol, copolymers of ethylene glycol/propylene glycol, ethylene/maleic anhydride copolymer, amino acid copolymers, copolymers of polyethylene glycol and an amino acid, polypropylene oxide/ethylene oxide copolymers, and polyethylene glycol/thiomalic acid copolymers.

5. The carrier of claim 1, wherein said polymer has a molecular weight ranging from about 1,000 to about 1,000,000 Daltons.

6. The carrier of claim 5, wherein said polymer has a molecular weight of about 20,000 to about 200,000 Daltons.

7. The carrier of claim 1, wherein said multiple thiol compounds are attached to said polymer at an interval.

8. The carrier of claim 7, wherein said interval is about 100 to about 10,000 Daltons.

9. The carrier of claim 1, wherein said polymer comprises multiple functional groups attached to said polymer and available for reaction with said thiol compound, so that said multiple thiol compounds are conjugated to said multiple functional groups.

10. The carrier of claim 9, wherein said multiple functional groups are attached to said polymer at an interval.

11. The carrier of claim 10, wherein said interval is about 100 to about 10,000 Daltons.

12. The carrier of claim 11, wherein said functional group comprises a ketone, an ester, a carboxylic acid, an aldehyde, an alcohol, a thiol, or an amine.

13. The carrier of claim 1, wherein said thiol compound comprises cysteamine, 1-amino-2-methyl-2-propanethiol, or 1-amino-2-propanethiol.

14. The carrier of claim 1, further comprising a cell uptake promoter conjugated to said polymer.

15. The carrier of claim 1, wherein said cell uptake promoter is biotin.

16. A conjugate for in-vivo delivery of a therapeutic agent comprising a thiol group, wherein said conjugate comprises:

a) a polymer;

b) multiple thiol compounds each comprising a sulfur atom conjugated to said polymer such that said sulfur atom of said thiol compound and said thiol group of said therapeutic agent can form a disulfide bond: and

c) a therapeutic agent comprising a thiol group bound to said polymer through said disulfide bond, wherein said therapeutic agent comprising a thiol group, further comprises a cell uptake promoter conjugated thereto.

17. The conjugate of claim 16, wherein said cell uptake promoter conjugated to said therapeutic agent is biotin.

18. The conjugate of claim 1, wherein said therapeutic agent comprising a thiol group comprises a Tat-inhibitory polypeptide, comprising an amino acid sequence of formula I:

R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-Cys-NH_2 (SEQ ID NO:1), biologically or pharmaceutically acceptable salts thereof, or stereo, optical or geometrical isomers thereof, as well as the pharmaceutically acceptable salts or solvates thereof, wherein

R comprises the residue of a carboxylic acid or an acetyl group; and

X is a Cys or Lys residue.

19. The conjugate of claim 18, wherein said therapeutic agent comprising a thiol compound comprises an amino acid sequence of:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:2)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-biotin)-Cys-NH_2 (SEQ ID NO:4)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO: 5)

N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:6) or

N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:7).

20. The conjugate of claim 1, wherein said therapeutic agent comprising a thiol group comprises a Tat-inhibitory polypeptide, comprising an amino acid sequence of SEQ ID NO:8:

N-acetyl-DCys-DLys-(biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-NH_2, or biologically or pharmaceutically acceptable salts thereof.

21. A method of making a carrier for in-vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer and multiple disulfide compounds conjugated thereto, each said disulfide compound comprising a first sulfur atom conjugated to a sulfur atom of a stable leaving group such that said first sulfur atom and the thiol group of the therapeutic agent can form a disulfide bond, wherein the method comprises the steps of:

a) reacting a thiol compound with a second disulfide compound comprising two stable leaving group to form a first intermediate wherein a sulfur atom of the thiol compound and a sulfur atom of the disulfide bond of the second disulfide compound form a disulfide bond; and

b) reacting multiple said first intermediates with said polymer to form the carrier, wherein said multiple first intermediates are conjugated to the polymer, so that the sulfur atoms of the multiple disulfide compounds and the thiol group of the therapeutic agent can form a disulfide bond.

22. The method of claim 21, wherein the second disulfide compound of step (a) is symmetric.

23. The method of claim 22, wherein said disulfide compound is 2,2'-dithiodipyridine.

24. The method of claim 21, wherein the polymer comprises a branched or linear structure.

25. The method of claim 21, wherein the polymer is a water soluble polymer.

26. The method of claim 25, wherein the polymer comprises polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, amino acid homopolymers, polypropylene glycol, copolymers of ethylene glycol/propylene glycol, ethylene/maleic anhydride copolymer, amino acid copolymers, copolymers of polyethylene glycol and an amino acid, polypropylene oxide/ethylene oxide copolymers, or polyethylene glycol/thiomalic acid copolymers.

27. The method of claim 21, wherein the polymer has a molecular weight ranging from about 1,000 to about 1,000,000 Daltons.

28. The method of claim 27, wherein the polymer has a molecular weight of about 20,000 to about 200,000 Daltons.

29. The method of claim 21, wherein the multiple disulfide compounds are conjugated to the polymer at an interval.

30. The method of claim 29, wherein the interval is about 100 to about 10,000 Daltons.

31. The method of claim 21, wherein the thiol compound comprises cysteamine, 1-amino-2-methyl-2-propanethiol, or 1-amino-2-propanethiol.

32. The method of claim 21, further comprising the step of conjugating a cell uptake promoter to the polymer prior to reacting the polymer with the first intermediate.

33. The method of claim 32, wherein said cell uptake promoter is biotin.

34. The method of claim 21, wherein said polymer comprises multiple functional groups attached thereto available for reaction, and step b) comprises reacting the first intermediate with the polymer comprising multiple functional groups to form the carrier, wherein the first intermediate is conjugated to the multiple functional groups attached to the polymer.

35. The method of claim 34, wherein the multiple functional groups are attached to the polymer at an interval.

36. The method of claim 35, wherein the interval is about 100 to about 10,000 Daltons.

37. The method of claim 35, wherein the multiple functional groups comprise a ketone, an ester, a carboxylic acid, and aldehyde, an alcohol, a thiol, or an amine.

38. The method of claim 21, wherein a cell uptake promoter is conjugated to the therapeutic agent.

39. The method of claim 38, wherein said cell uptake promoter is biotin.

40. The method of claim 39, wherein said therapeutic agent comprising a thiol group comprises a Tat-inhibitory polypeptide comprising a polypeptide of the formula I:

R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin) Cys-NH_2 (SEQ ID NO:1), biologically or pharmaceutically acceptable salts thereof, or stereo, optical or geometrical isomers thereof or pharmaceutically acceptable salts thereof, wherein

R comprises the residue of a carboxylic acid or an acetyl group; and

X is a Cys or Lys residue.

41. The method of claim 40, wherein said therapeutic agent comprises an amino acid sequence of:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ IDNO:2)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:4)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:5)

N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:6) or

N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys (biotin)-Cys-NH_2 (SEQ ID NO:7).

42. The method of claim 39, wherein said therapeutic agent comprises a Tat-inhibitory binding protein comprising an amino acid sequence of SEQ ID NO:8:

N-acetyl-DCys-DLys-(biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-NH_2, or biologically or pharmaceutically acceptable salts thereof.

43. A method of making a carrier for in-vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises a polymer and multiple disulfide compounds conjugated thereto, each said disulfide compound comprising a first sulfur atom conjugated to a sulfur atom of a stable leaving group such that said first sulfur atom and the thiol group of the therapeutic agent can form a disulfide bond, wherein the method comprises the steps of:

a) reacting a first thiol compound with a second thiol compound to form a first intermediate, wherein a thiol group of the first thiol compound and a thiol group of the second thiol compound form a disulfide bond;

b) reacting multiple said first intermediates with a polymer to form a second intermediate, wherein multiple said first intermediates are conjugated to the polymer;

c) reducing the disulfide bond of the second intermediate to form a third intermediate comprising the polymer and the multiple first thiol compounds conjugated thereto so that the thiol groups of the first thiol compounds are is available for reaction; and

d) reacting the third intermediate with a disulfide compound comprising two stable leaving groups to form the carrier, wherein the sulfur atom of the first thiol compound and a sulfur atom of the a leaving group form a disulfide bond, so that the sulfur atom of the first thiol compound and the thiol group of the therapeutic agent can form a disulfide bond.

44. The method of claim 43, wherein the disulfide compound of step (d) is symmetric.

45. The method of claim 44, wherein said disulfide compound is 2,2'-dithiodipyridine.

46. The method of claim 43, wherein the polymer comprises a branched or linear structure.

47. The method of claim 43, wherein the polymer is a water soluble polymer.

48. The method of claim 47, wherein the polymer comprises polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, amino acid homopolymers, polypropylene glycol, copolymers of ethylene glycol/propylene glycol, ethylene/maleic anhydride copolymer, amino acid copolymers, copolymers of polyethylene glycol and an amino acid, polypropylene oxide/ethylene oxide copolymers, or polyethylene glycol/thiomalic acid copolymers.

49. The method of claim 43, wherein the polymer has a molecular weight ranging from about 1,000 to about 1,000,000 Daltons.

50. The method of claim 49, wherein the polymer has a molecular weight of about 20,000 to about 200,000 Daltons.

51. The method of claim 43, wherein the multiple disulfide compounds are conjugated to the polymer at an interval.

52. The method of claim 51, wherein the interval is about 100 to about 10,000 Daltons.

53. The method of claim 43, wherein the polymer comprises multiple functional groups attached thereto, and step b) comprises reacting the first intermediate with the polymer comprising multiple functional groups attached thereto to form a second intermediate, wherein the first intermediate is conjugated to the multiple functional groups.

54. The method of claim 53, wherein the multiple functional groups are attached to the polymer at an interval.

55. The method of claim 54, wherein the interval is about 100 to about 10,000 Daltons.

56. The method of claim 54, wherein the multiple functional groups comprise a ketone, an ester, a carboxylic acid, an aldehyde, an alcohol, a thiol, or an amine.

57. The method of claim 43, wherein the thiol compound comprises cysteamine, 1-amino-2-methyl-2-propanethiol, or 1-amino-2-propanethiol.

58. The method of claim 43, further comprising the step of conjugating a cell uptake promoter to the polymer prior to reacting the polymer with the first intermediate.

59. The method of claim 58, wherein said cell uptake promoter is biotin.

60. A carrier for in-vivo delivery of a therapeutic agent comprising a thiol group, wherein said carrier comprises:

a) a polymer;

b) multiple thiol compounds conjugated to said polymer, said thiol compounds comprising a sulfur atom and at least one functional group; and

c) a cell uptake promoter conjugated to said polymer, such that said sulfur atom of said thiol compounds and said thiol group of said therapeutic agent can form a disulfide bond, and the rate at which the disulfide bond is reduced in vivo so that said therapeutic agent is released from the carrier, is dependent on the size of the functional group attached to the thiol compound.

61. A method of treating a viral infection in a mammal, comprising administering to the mammal a therapeutically effective amount of a carrier for in-vivo delivery of a therapeutic agent comprising a thiol group, wherein the carrier comprises:

a) a polymer; and

b) at least one thiol compound conjugated to said polymer such that said thiol group of said thiol compound and said thiol group of said therapeutic agent form a disulfide bond, and

wherein the therapeutic agent is a Tat-inhibitory polypeptide.

62. The method of claim 61, wherein said Tat-inhibitory polypeptide derivative comprises:

R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-Cys-NH_2 (SEQ ID NO:1), or pharmaceutically acceptable salts thereof, or stereo, optical or geometrical isomers thereof or pharmaceutically acceptable salts or solvates thereof, wherein

R comprises the residue of a carboxylic acid or an acetyl group; and

X is a Cys or Lys residue.

63. The method of claim 62, wherein said Tat-inhibitory polypeptide derivative comprises an amino acid sequence of:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2(SEQ IDNO:2)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2(SEQ ID NO:3)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-(biotin)-Cys-NH_2(SEQ ID NO :4)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2(SEQ ID NO: 5)

N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2(SEQ ID NO:6) or

N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys (biotin)-Cys-NH_2(SEQ ID NO:7).

64. The method of claim 61, wherein said Tat-inhibitory polypeptide comprises an amino acid sequence of SEQ ID NO:8:

N-acetyl-DCys-DLys-(biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-NH_2 or biologically or pharmaceutically acceptable salts thereof.

65. The method of any of claims 61, 62,63, or 64, wherein said viral infection is Aids.

66. The method of claim 65, wherein said mammal comprises humans.

67. The carrier of claim 6 wherein said polymer has a molecular weight of about 27,000 Daltons.

68. The carrier of claim 8 wherein said interval is about 300 to about 3,000 Daltons.

69. The carrier of claim 11 wherein said interval is about 300 to about 3,000 Daltons.

70. The method of claim 28 wherein the polymer has a molecular weight of about 27,000 Daltons.

71. The method of claim 30 wherein said interval is about 300 to about 3,000 Daltons.

72. The method of claim 36 wherein said interval is about 300 to about 3,000 Daltons.

73. The method of claim 50 wherein the polymer has a molecular weight of about 27,000 Daltons.

74. The method of claim 52 wherein said interval is about 300 to about 3,000 Daltons.

75. The method of claim 55 wherein said interval is about 300 to about 3,000 Daltons.

76. The method of claim 1 wherein said therapeutic agent comprising a thiol group is a Tat inhibitory peptide.

77. The conjugate of claim 16, wherein said polymer comprises a branched or linear structure.

78. The conjugate of claim 16, wherein said polymer is a water soluble polymer.

79. The conjugate of claim 78, wherein said water soluble polymer is selected from the group consisting of polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6trioxane, amino acid homopolymers, polypropylene glycol, copolymers of ethylene glycol/propylene glycol, ethylene/maleic anhydride co polymer, amino acid copolymers, copolymers of polyethylene glycol and an amino acid, polypropylene oxide/ethylene oxide copolymers, and polyethylene glycol/thiomalic acid copolymers.

80. The conjugate of claim 16, wherein said polymer has a molecular weight ranging from about 1,000 to about 1,000,000 Daltons.

81. The conjugate of claim 80, wherein said polymer has a molecular weight of about 20,000 to about 200,000 Daltons.

82. The conjugate of claim 81 wherein said polymer has a molecular weight of about 27,000 Daltons.

83. The conjugate of claim 16, wherein said multiple thiol compounds are attached to said polymer at an interval.

84. The conjugate of claim 83, wherein said interval is about 100 to about 10,000 Daltons.

85. The conjugate of claim 84 wherein said interval is about 300 to about 3,000 Daltons.

86. The conjugate of claim 16, wherein said polymer comprises multiple functional groups attached to said polymer and available for reaction with said thiol compound, so that said multiple thiol compounds are conjugated to said multiple functional groups.

87. The conjugate of claim 86 wherein said multiple functional groups are attached to said polymer at an interval.

88. The conjugate of claim 87, wherein said interval is about 100 to about 10,000 Daltons.

89. The conjugate of claim 86, wherein said functional group comprises a ketone, an ester, a carboxylic acid, an aldehyde, an alcohol, a thiol, or an amine.

90. The conjugate of claim 16, wherein said thiol compound comprises cysteamine, 1-amino-2-methyl-2-propanethiol, or 1-amino-2-propanethiol.

91. A conjugate for in-vivo delivery of a therapeutic agent comprising a thiol group, wherein said conjugate comprises:

a) a polymer and a cell uptake promoter conjugated thereto;

b) multiple thiol compounds each comprising a sulfur atom conjugated to said polymer such that said sulfur atom of said thiol compound and said thiol group of said therapeutic agent can form a disulfide bond; and

c) a therapeutic agent comprising a thiol group bound to said polymer through said disulfide bond.

92. The conjugate of claim **91**, wherein said polymer comprises a branched or linear structure.

93. The conjugate of claim **91**, wherein said polymer is a water soluble polymer.

94. The conjugate of claim **93**, wherein said water soluble polymer is selected from the group consisting of polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1, 3,6-trioxane, amino acid homopolymers, polypropylene glycol, copolymers of ethylene glycol/propylene glycol, ethylene/maleic anhydride copolymer, amino acid copolymers, copolymers of polyethylene glycol and an amino acid, polypropylene oxide/ethylene oxide copolymers, and polyethylene glycol/thiomalic acid copolymers.

95. The conjugate of claim **91**, wherein said polymer has a molecular weight ranging from about 1,000 to about 1,000,000 Daltons.

96. The conjugate of claim **95**, wherein said polymer has a molecular weight of about 20,000 to about 200,000 Daltons.

97. The conjugate of claim **96** wherein said polymer has a molecular weight of about 27,000 Daltons.

98. The conjugate of claim **91**, wherein said multiple thiol compounds are attached to said polymer at an interval.

99. The conjugate of claim **98**, wherein said interval is about 100 to about 10,000 Daltons.

100. The conjugate of claim **99** wherein said interval is about 300 to about 3,000 Daltons.

101. The conjugate of claim **91**, wherein said thiol compound comprises cysteamine, 1-amino-2-methyl-2-propanethiol, or 1-amino-2-propanethiol.

102. The conjugate of claim **91**, wherein said polymer comprises multiple functional groups attached to said polymer and available for reaction with said thiol compound, so that said multiple thiol compounds are conjugated to said multiple functional groups.

103. The conjugate of claim **102** wherein said multiple functional groups are attached to said polymer at an interval.

104. The conjugate of claim **103**, wherein said interval is about 100 to about 10,000 Daltons.

105. The conjugate of claim **104**, wherein said functional group comprises a ketone, an ester, a carboxylic acid, an aldehyde, an alcohol, a thiol, or an amine.

106. The conjugate of claim **91**, wherein said cell uptake promoter is biotin.

107. The conjugate of claim **91**, wherein said therapeutic agent comprising a thiol group comprises a Tat-inhibitory polypeptide, comprising an amino acid sequence of formula I: R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-Cys-NH_2 (SEQ ID NO: 1), biologically or pharmaceutically acceptable salts thereof, or stereo, optical or geometrical isomers thereof, as well as the pharmaceutically acceptable salts or solvates thereof, wherein

R comprises the residue of a carboxylic acid or an acetyl group; and

X is a Cys or Lys residue.

108. The conjugate of claim **91**, wherein said therapeutic agent comprising a thiol compound comprises an amino acid sequence of:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO: 2)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:3)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:4)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:5)

N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-Cys-NH_2 (SEQ ID NO:6) or

N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys-(biotin)-Cys-NH_2 (SEQ ID NO:7).

109. The conjugate of claim **91**, wherein said therapeutic agent comprising a thiol group comprises a Tat-inhibitory polypeptide, comprising an amino acid sequence of SEQ ID NO:8:

N-acetyl-DCys-DLys-(biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-NH_2

or biologically or pharmaceutically acceptable salts thereof.

110. A method for intracellular delivery of a therapeutic agent comprising a thiol group comprising contacting a cell with an effective intracellular delivery promoting amount of a conjugate of claim **16**.

111. A method for intracellular delivery of a therapeutic agent comprising a thiol group comprising contacting a cell with an effective intracellular delivery promoting amount of a conjugate of claim **91**.

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